VOLUME 36 APRIL 1958 NUMBER 4

Canadian Journal of Biochemistry and Physiology

Editor: K. A. C. ELLIOTT

Associate Editors:

B. D. BURNS, *McGill University*
L.-P. DUGAL, *University of Ottawa*
G. KROTKOV, *Queen's University*
A. G. McCALLA, *University of Alberta*
J. A. McCARTER, *Dalhousie University*
M. NICKERSON, *University of Manitoba*
H. E. RAWLINSON, *University of Alberta*
R. J. ROSSITER, *University of Western Ontario*
A. E. WILHELMI, *Emory University*

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

CANADIAN JOURNAL OF BIOCHEMISTRY AND PHYSIOLOGY

(Formerly Canadian Journal of Medical Sciences)

Under the authority of the Chairman of the Committee of the Privy Council on Scientific and Industrial Research, the National Research Council issues THE CANADIAN JOURNAL OF BIOCHEMISTRY AND PHYSIOLOGY and five other journals devoted to the publication, in English or French, of the results of original scientific research. *La Revue accepte des travaux originaux en biochimie, physiologie, pharmacologie, et sujets connexes.*

Matters of general policy concerning these journals are the responsibility of a joint Editorial Board consisting of: members representing the National Research Council of Canada; the Editors of the Journals; and members representing the Royal Society of Canada and four other scientific societies. The Canadian Physiological Society has chosen the Canadian Journal of Biochemistry and Physiology as its official journal for the publication of scientific papers.

EDITORIAL BOARD

Representatives of the National Research Council

R. B. MILLER, *University of Alberta*
H. G. THODE, *McMaster University*
D. L. THOMSON, *McGill University*
W. H. WATSON (Chairman), *University of Toronto*

Editors of the Journals

D. L. BAILEY, *University of Toronto*
T. W. M. CAMERON, *Macdonald College*
H. E. DUCKWORTH, *McMaster University*
K. A. C. ELLIOTT, *Montreal Neurological Institute*
LÉO MARION, *National Research Council*
R. G. E. MURRAY, *University of Western Ontario*

Representatives of Societies

D. L. BAILEY, *University of Toronto*
Royal Society of Canada
T. W. M. CAMERON, *Macdonald College*
Royal Society of Canada
H. E. DUCKWORTH, *McMaster University*
Royal Society of Canada
Canadian Association of Physicists
K. A. C. ELLIOTT, *Montreal Neurological Institute*
Canadian Physiological Society
R. G. E. MURRAY, *University of Western Ontario*
Canadian Society of Microbiologists
H. G. THODE, *McMaster University*
Chemical Institute of Canada
T. THORVALDSON, *University of Saskatchewan*
Royal Society of Canada

Ex officio

LÉO MARION (Editor-in-Chief), *National Research Council*
J. B. MARSHALL, Division of Administration and Awards, *National Research Council*

Manuscripts for publication should be submitted to Dr. K. A. C. Elliott, Editor, Canadian Journal of Biochemistry and Physiology, Montreal Neurological Institute, 3801 University Street, Montreal, Canada.

(For instructions on preparation of copy, see **Notes to Contributors** (inside back cover).)

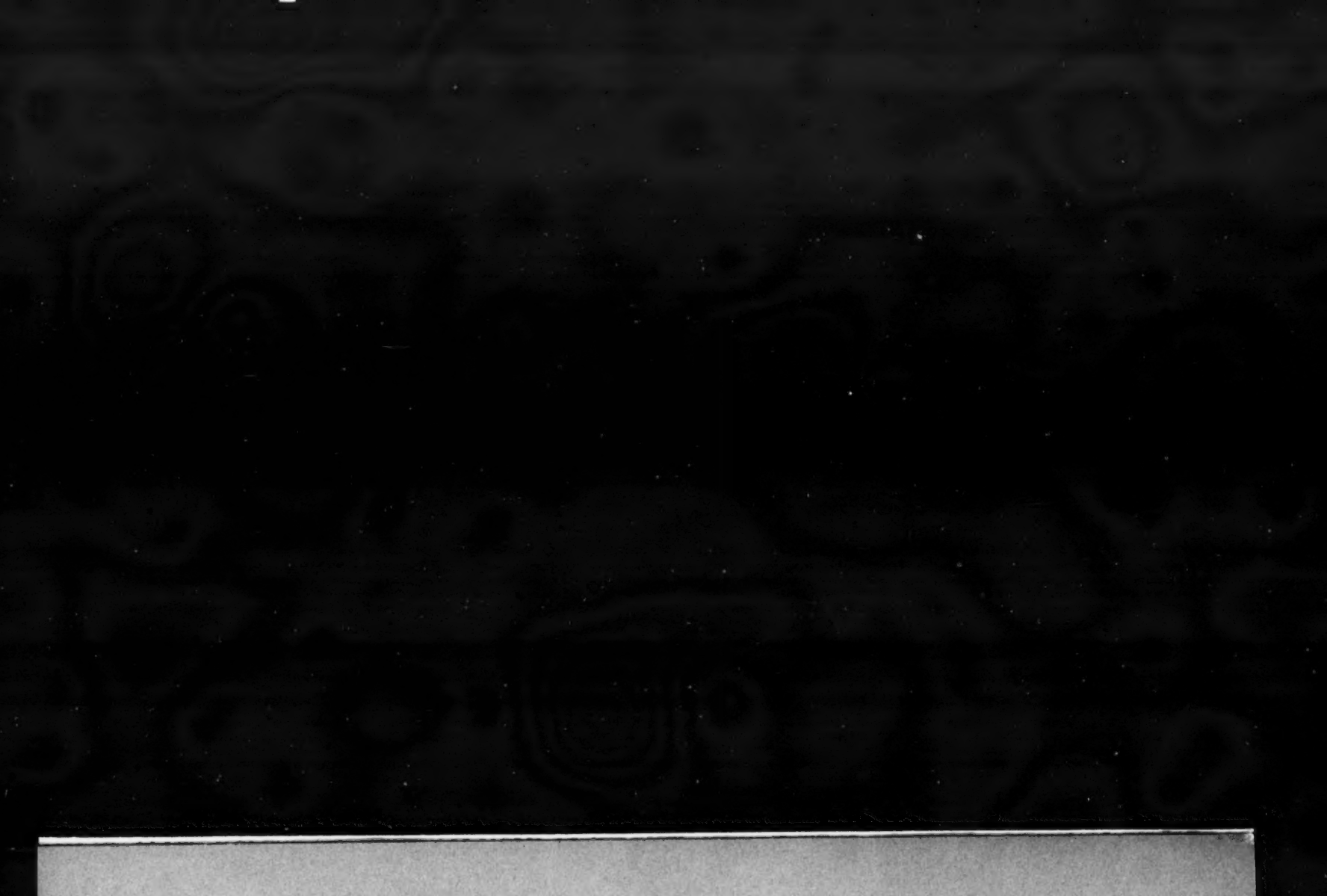
Proof, correspondence concerning proof, and orders for reprints should be sent to the Manager, Editorial Office (Research Journals), Division of Administration and Awards, National Research Council, Ottawa 2, Canada.

Subscriptions, renewals, requests for single or back numbers, and all remittances should be sent to Division of Administration and Awards, National Research Council, Ottawa 2, Canada. Remittances should be made payable to the Receiver General of Canada, credit National Research Council.

The journals published, frequency of publication, and prices are:

Canadian Journal of Biochemistry and Physiology	Monthly	$3.00 a year
Canadian Journal of Botany	Bimonthly	$4.00
Canadian Journal of Chemistry	Monthly	$5.00
Canadian Journal of Microbiology	Bimonthly	$3.00
Canadian Journal of Physics	Monthly	$4.00
Canadian Journal of Zoology	Bimonthly	$3.00

The price of regular single numbers of all journals is 75 cents.



Canadian Journal of Biochemistry and Physiology

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOLUME 36 APRIL 1958 NUMBER 4

STUDIES ON WHEAT PLANTS USING C^{14} COMPOUNDS

VII. UTILIZATION OF PYRUVATE-2-C^{14} [1]

E. BILINSKI[2] AND W. B. MCCONNELL

Abstract

Approximately half of the carbon-14 injected into the stems of wheat plants in the form of pyruvate-2-C^{14} remained in the plant at maturity, 30 days later. Almost 90% of this had accumulated in the kernel. Appreciable activity was found in the major components, protein, starch, ether-soluble material, and a residue termed bran. The amino acids of the gluten protein differed markedly from one another in specific activity. Glutamic acid and the related amino acids, arginine and proline, were most active, their specific activity decreasing in that order. Fifty-two per cent of the carbon-14 in glutamic acid was in carbon-5, while carbon-1 contained 21%. Seventy per cent of the radioactivity of aspartic acid was divided almost equally between the terminal carboxyl groups. The results are similar to those previously observed using acetate-1-C^{14} as tracer, and it is concluded that administered pyruvate-2-C^{14} undergoes extensive decarboxylation to form acetate-1-C^{14}. The most active carbon in alanine from the pyruvate-2-C^{14} was carbon-1. This observation is not in accord with the theory that alanine is formed directly from pyruvate by transamination.

Introduction

Papers III and IV (1, 2) of this series describe the incorporation of carbon-14 from administered acetate-1-C^{14} into the amino acids of gluten in mature wheat plants. The pattern of varying specific activity from one amino acid to another suggested that much of the acetate is utilized by way of the tricarboxylic acid (T.C.A.) cycle (1). The importance of this cycle as a biosynthetic pathway in maturing wheat was confirmed by a study of the intramolecular distribution of C^{14} in the dicarboxylic amino acids (2); localization of C^{14} in the carbon skeleton of these amino acids was quite specific and in accord with the pattern to be expected from the T.C.A. cycle if some recycling of C^{14} occurred.

These studies are parallel to recent investigations of Black and Kleiber (3, 4) on the incorporation of acetate-C^{14} into the amino acids of the milk protein, casein, following administration of labelled acetate to the lactating cow. The similarity of labelling in the dicarboxylic acids isolated from wheat gluten

[1]Manuscript received October 25, 1957.
Contribution from the National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan. Presented in part at the Fortieth Annual Conference of the Chemical Institute of Canada at Vancouver, British Columbia, June, 1957.
Issued as N.R.C. No. 4601.
[2]Postdoctorate Fellow, National Research Council of Canada, 1955–1957.

(2) and from casein is striking and may be due, in part at least, to the fact that both gluten and casein are reserve or storage proteins which, once formed in the organism, probably undergo further change slowly. It is thus feasible to use long term tracer experiments to study certain biochemical processes related to the biosynthesis of this class of proteins (5, 6).

The utilization of C^{14} from pyruvate-2-C^{14} by wheat plants has now been investigated. Good incorporation of tracer was observed and a striking parallelism was noted between results obtained with pyruvate-2-C^{14} and those reported previously for acetate-1-C^{14} (2).

Experimental Methods

Production of Wheat Labelled with C^{14}

Thatcher wheat was sown in a field plot on May 17, 1956, and harvested fully mature on August 29, the total growing period being 104 days. Growth conditions were favorable and there was no appreciable damage to plants by insects or disease; the grain graded No. 1 Northern. Fifteen typical plants were labelled on July 30 (74 days after seeding) by injecting into the stem of each 0.1 ml. of a solution containing 5 μc. of sodium pyruvate-2-C^{14} (0.17 mg.). The plants were air-dried after harvesting; separated into stems, rachis, chaff, and kernels; and further vacuum-dried for 48 hours at 40° C. The dried tissues were ground to pass a 40-mesh screen. The wheat kernels were fractionated as in previous experiments (5) into lipids, starch, bran, albumin, and gluten.

Determination of Specific Activity

Radioactive preparations were converted to carbon dioxide by wet combustion and the specific activity of the gas was determined (2, 7). The specific activities are expressed as millimicrocuries of C^{14} per millimole of carbon dioxide (mμc./mM.CO_2).

Isolation of Amino Acids

Gluten (500 mg.) was hydrolyzed by refluxing for 24 hours in 100 ml. of 6 *N* hydrochloric acid. The hydrolyzate was freed of hydrochloric acid and humin, and the amino acids were isolated by successive chromatography on columns of Dowex 1×8 (60 cm. long) and Dowex 50×8 (150 cm. long) following the method of Hirs, Moore, and Stein (8). Minor modifications of the method have been described previously (1).

Degradation of Amino Acids

The amino acids were partially degraded by decarboxylation according to the method of Van Slyke, MacFayden, and Hamilton (9) for the titrimetric determination of carbon dioxide liberated from amino acids by ninhydrin. Carbon dioxide liberated by this reaction was collected in sodium hydroxide solution prior to counting as previously described (1).

The specific activities of the terminal carbons of glutamic acid were determined by converting them to carbon dioxide. Carbon-1 was obtained by

using ninhydrin and carbon-5 using the Schmidt reaction (10, 11). Aspartic acid and threonine were degraded using previously developed methods which have been described in detail elsewhere (2). Alanine was degraded according to the method described by Högström (12). Serine was reduced to alanine with concentrated hydriodic acid and red phosphorus and the alanine degraded as above (13).

Experimental Results and Discussion

The distribution of the weight and of the C^{14} in the wheat plants given pyruvate-2-C^{14} is shown in Fig. 1. The kernels account for about 66% of the dry weight of the plant parts above the top node of the stem and contain all but about 11% of the C^{14}. Thus C^{14} was preferentially deposited in the kernels whereas the stems into which the tracer was originally injected contained only 6% of the C^{14} in the mature plants.

The distribution of kernel components and the amount of radioactivity each contained is shown in Fig. 2. The material designated as "water soluble" contains substances discarded with the various waste solutions involved in the fractionation (5); its weight and total radioactivity were estimated by difference. Sixty per cent of the kernel weight was isolated as starch and this contained about 45% of the C^{14} in the kernels. The specific activities of the main constituents as isolated are as follows: gluten 27, starch 54, albumin 50, ether soluble material 47, and bran 38 mμc./mM.CO_2. Gluten was thus the most active substance isolated but the tracer was more uniformly distributed among the kernel components than was observed with the earlier experiments using acetate-C^{14}. In particular starch had incorporated relatively more pyruvate carbon than acetate carbon. Thus gluten was about three and one-half times as active as starch using acetate-1-C^{14} and more than seven times as active as starch with acetate-2-C^{14} (1). It is noted, however, that the relative labelling of different components depends upon the stage of growth when injection is made (5, 6) and since pyruvate and acetate were used on different dates of different years the results are not strictly comparable.

The specific activities of amino acids isolated from the gluten are given in Table I together with the specific activities of their respective carboxyl carbons. Results previously obtained by feeding acetate-1-C^{14} are included for purposes of comparison and, since pyruvate-2-C^{14} was administered at the rate of 5 μc./plant, the values for acetate-1-C^{14} have been adjusted accordingly by use of the appropriate factor. Although there are quantitative differences between the results with the two tracers there are many qualitative similarities. Glutamic acid, for example, is the most active amino acid isolated and the closely related amino acids, arginine and proline, are next most radioactive. As expected, the short-chain amino acids, glycine, alanine, and serine, are moderately active following pyruvate injection. However, alanine possesses less C^{14} than should be found if it were derived directly from pyruvate by transamination. Furthermore, the high activity of carbon-1 of alanine is not consistent with the view that it is derived directly from pyruvate. The

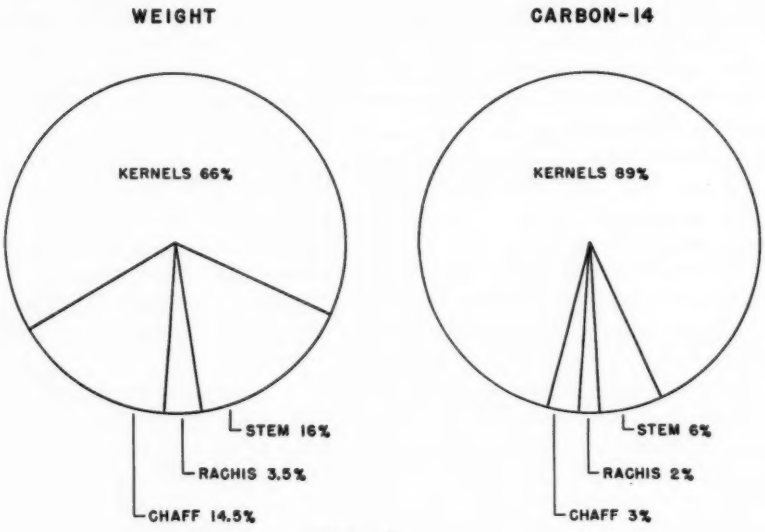


FIG. 1

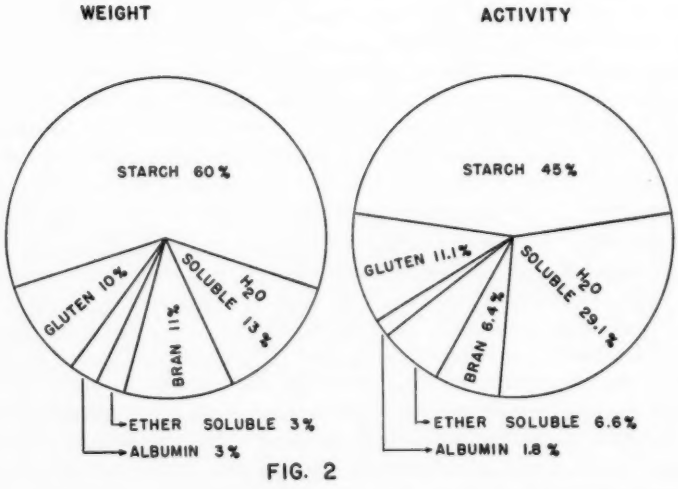


FIG. 2

FIG. 1. Distribution of weight and of carbon-14 in wheat plants injected with pyruvate-2-C^{14}. Fifty-six per cent of the C^{14} injected remained in the mature plants.

FIG. 2. Distribution of weight and of carbon-14 in the components of the wheat kernels. The mature kernels contained 50% of the C^{14} injected as pyruvate-2-C^{14}.

TABLE I

SPECIFIC ACTIVITY OF AMINO ACIDS

Amino acid	Specific activity ($m\mu c./mM.CO_2$)			
	Pyruvate-2-C^{14}		Acetate-2-C^{14}*	
	Total	1-C	Total	1-C
Glutamic acid	163	171	310	310
Arginine	122	141	125	127
Proline	102	151	181	240
Aspartic acid	52	79	72	112
Threonine	46	56	48	83
Alanine	49	59	39	38
Serine	61	74	56	62
Glycine	73	69	74	67
Methionine	70	119	—	—
Leucine	22	43	25	71
Isoleucine	25	37	11	13
Valine	24	19	10	17
Histidine	13	22	12	6
Lysine	25	32	15	18
Tyrosine	51	25	35	41
Phenylalanine	41	38	25	30

*Calculated from Table II of Reference (1).

branched-chain amino acids, leucine, isoleucine, and valine, are not strongly labelled by either tracers. However, leucine possesses the highest ratio of specific activity of carbon-1 to total activity for both tracers which indicates direct incorporation of the labelled carbon into the carboxyl groups of this amino acid (1). Histidine and lysine are weakly labelled with both tracers, the most notable difference between the tracers being in the specific activity of the carboxyl group of histidine. Pyruvate-2-C^{14} is more effective than acetate for labelling both tyrosine and phenylalanine. It was observed further that the carboxyl group of these amino acids is less radioactive than are the acids as a whole. This observation is in accord with previous observations with yeast (14) and it is probable that the intact pyruvic acid molecule has been incorporated into the side chain of these amino acids.

The similarity of the labelling with pyruvate-2-C^{14} and with acetate-1-C^{14} was further demonstrated by the results of partial degradation of aspartic acid and glutamic acid (Fig. 3). The values quoted for carbons 2, 3, and 4 of glutamic acid are calculated by difference on the assumption that all have the same specific activity. Although this assumption is undoubtedly not strictly valid it is probable that, as found earlier with acetate-1-C^{14}, their activities are of the same order of magnitude and that all are less active than either

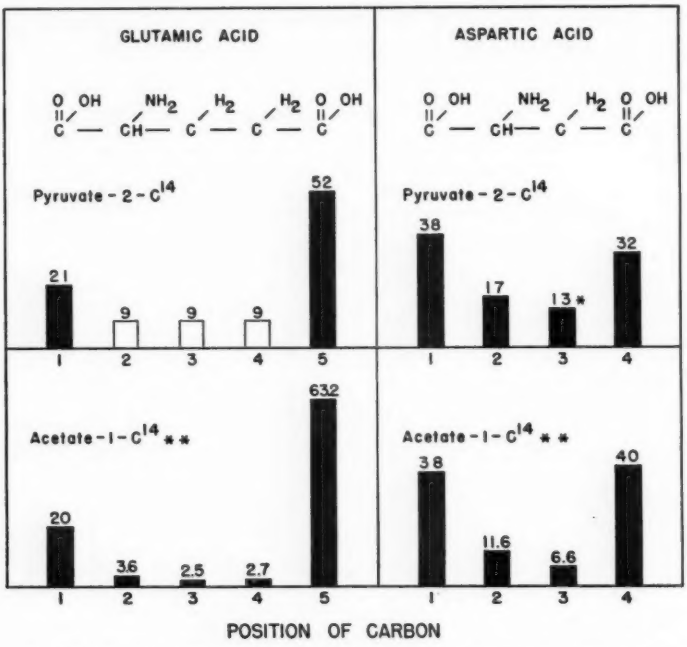


FIG. 3. Distribution of carbon-14 in glutamic acid and aspartic acid. *Calculated by difference. **From reference 1.

carbons 1 or 5. The specific activities of carbons 1, 2, and 4 of aspartic acid were determined as described previously (2); carbon-3 activity was obtained by difference.

The labelling pattern in these amino acids can be best explained by assuming that they are formed by way of the tricarboxylic acid cycle, the pyruvate-2-C^{14} undergoing preliminary decarboxylation to form acetate-1-C^{14}. Such a reaction would explain not only the amount of radioactivity and its distribution in glutamic and aspartic acids but would account for the close qualitative agreement between results found with acetate-1-C^{14} and pyruvate-2-C^{14} for the other amino acids. The labelling in glutamic acid is similar to that reported for yeast grown with pyruvate-2-C^{14} by Wang, Christensen, and Cheldelin (15), who found carbons 1 and 5 contained 27% and 50% respectively of the C^{14} in the glutamic acid. These authors interpreted the result as indicating incorporation of the tracer by way of the tricarboxylic acid cycle. The labelling of aspartic acid isolated from wheat in the present experiments is considerably different from that of yeast grown with pyruvate-2-C^{14}, as reported by Wang, Thomas, Cheldelin, and Christensen (16). Since the latter workers found considerable labelling in the amino carbon they suggested that the Wood–Werkman (17) or similar reaction was operative to a considerable extent in yeast. The present experiments with wheat plants gave labelling

predominately in the terminal carbons of aspartic acid. This is as observed following administration of acetate-1-C^{14} (2) and is in accord with the previous suggestion that decarboxylation of pyruvate to acetate is a major reaction. If condensation of the Wood–Werkman type occurred in the wheat plants it probably plays a minor role.

The distribution of C^{14} in the carbon skeleton of alanine is difficult to explain (Fig. 4). Formation of alanine from pyruvate by the well-known transamination reaction should have given alanine-2-C^{14} of comparatively high activity (18), but in the present experiments carbon-1 of alanine was the most active. It appears either that transamination to form alanine from pyruvate took place slowly compared to other reactions of the pyruvate, or that if alanine was formed initially from the tracer by transamination it underwent extensive further reaction before incorporation into stable protein. The latter possibility seems unlikely and additional investigation of the formation of alanine of wheat gluten is necessary. The present authors found earlier (1) that the labelling of alanine formed in wheat from acetate-1-C^{14} and -2-C^{14} was not consistent with the idea that it was derived from pyruvate in equilibrium with oxalacetate.

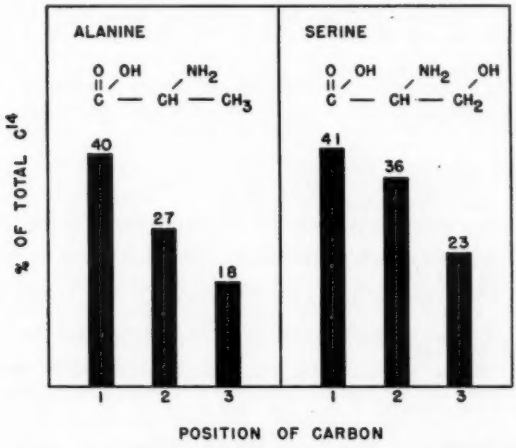


FIG. 4. Distribution of carbon-14 in alanine and serine.

The labelling in serine is similar to that of alanine in that carbons 1 and 2 are most active. This result is, however, in better agreement with previous studies and with the idea that direct conversion of the carbon skeleton of pyruvate to serine does not occur. Yeast, for example (18), grown either anaerobically or aerobically with pyruvate-2-C^{14}, contains serine with activity in both carbons 1 and 2. Although carbon-2 was somewhat more active than carbon-1, the result was interpreted as showing that the pathway must involve one or more steps that permit equilibration of carbons 1 and 2. Furthermore,

Nyc and Zabin (19) found that carbon-3 of pyruvate was distributed among carbons 2 and 3 of serine from rat tissues. Koeppe, Minthorn, and Hill (20) conclude that, in the rat, serine was formed more or less directly from a three-carbon intermediate of glycolysis other than pyruvate. It has been shown, on the other hand, that in mammalian tissue serine can be formed from condensation of a one-carbon fragment and a two-carbon fragment such as glycine (21). Although the present studies provide little information about serine formation in wheat, the concept of its biosynthesis by way of the above condensation has previously been indicated for plants (22) and is strongly supported by studies in this laboratory using formate-C^{14} and glycine-1-C^{14} (23).

Acknowledgment

The authors are indebted to Mr. M. Mazurek for able technical assistance and to Mr. J. Dyck for determination of specific activity.

References

1. Bilinski, E. and McConnell, W. B. Can. J. Biochem. and Physiol. **35**, 357 (1957).
2. Bilinski, E. and McConnell, W. B. Can. J. Biochem. and Physiol. **35**, 365 (1957).
3. Black, A. L., Kleiber, M., Smith, A. H., and Stewart, D. N. Biochim. et Biophys. Acta, **23**, 54 (1957).
4. Black, A. L. and Kleiber, M. Biochim. et Biophys. Acta, **23**, 59 (1957).
5. McConnell, W. B. and Ramachandran, L. K. Can. J. Biochem. and Physiol. **34**, 180 (1956).
6. Bilinski, E. and McConnell, W. B. Cereal Chem. In press.
7. Buchanan, D. L. and Nakao, A. J. Am. Chem. Soc. **74**, 2389 (1952).
8. Hirs, C. H. W., Moore, S., and Stein, W. H. J. Am. Chem. Soc. **76**, 6063 (1954).
9. Van Slyke, D. D., MacFayden, D. A., and Hamilton, P. J. Biol. Chem. **141**, 671 (1941).
10. Adamson, D. W. J. Chem. Soc. 1564 (1939).
11. Phares, E. F. Arch. Biochem. Biophys. **33**, 173 (1951).
12. Högström, G. Acta Chem. Scand. **7**, 45 (1953).
13. Ehrensvärd, G., Reio, L., Saluste, E., and Stjernholm, R. J. Biol. Chem. **189**, 93 (1951).
14. Thomas, R. C., Cheldelin, V. H., Christensen, B. E., and Wang, C. H. J. Am. Chem. Soc. **75**, 5554 (1953).
15. Wang, C. H., Christensen, B. E., and Cheldelin, V. H. J. Biol. Chem. **201**, 683 (1953).
16. Wang, C. H., Thomas, R. C., Cheldelin, V. H., and Christensen, B. E. J. Biol. Chem. **197**, 663 (1952).
17. Werkman, C. H. and Wood, H. G. Advances in enzymology. Vol. 2. *Edited by* F. F. Nord and C. H. Werkman. Interscience Publishers, New York. 1942. p. 135.
18. Wang, C. H., Christensen, B. E., and Cheldelin, V. H. J. Biol. Chem. **213**, 365 (1955).
19. Nyc, J. F. and Zabin, I. J. Biol. Chem. **215**, 35 (1955).
20. Koeppe, R. E., Minthorn, M. L., Jr., and Hill, R. J. Arch. Biochem. Biophys. **68**, 355 (1957).
21. Meister, A. Biochemistry of amino acids. Academic Press, New York. 1957.
22. Tolbert, N. E. and Cohen, M. S. J. Biol. Chem. **204**, 649 (1953).
23. Bilinski, E. and McConnell, W. B. Unpublished results.

SEPARATION AND CHARACTERIZATION OF LIPOVITELLIN FROM HEN EGG YOLK[1]

F. J. JOUBERT[2] AND W. H. COOK

Abstract

The major sedimenting fraction of egg yolk, which appears to be homogeneous in sodium chloride solutions, has been fractionated into lipovitellin, phosvitin, and γ-livetin. By dissolving egg yolk in 0.4 *M* magnesium sulphate and diluting to 0.2 *M* much of the phosvitin is precipitated and further dilution of the supernatant yields lipovitellin, from which most of the γ-livetin and contaminating phosvitin can be removed by further treatment. Separation and recovery of both lipovitellin and phosvitin by this procedure indicate that phosvitin is a separate protein and not an integral part of the lipovitellin molecule. When the sedimenting fraction in egg yolk is dissolved in buffer solutions at pH 9.0, γ-livetin can be resolved ultracentrifugally from the rest of this fraction, but precipitation by dilution, with ammonium sulphate, or with ethanol, failed to separate the components. Evidently lipovitellin as prepared in the past by similar methods has been a mixture of three proteins. Lipovitellin containing about 10% γ-livetin and 20% lipid had a molecular weight of 3.2×10^6 and a phosphorus content of 0.49%, about half that previously reported.

Introduction

When hen egg yolk is diluted with 10% sodium chloride solution, one rising and two sedimenting boundaries are observed on ultracentrifugation (1). On isolation, the major sedimenting fraction (S1), dissolved in 10% sodium chloride solution, appeared to be homogeneous in the ultracentrifuge and resembled lipovitellin in lipid content (2, 3). It was subsequently found that there were three livetins (globulins) (4) in egg yolk and that one of these (γ-livetin) could not be distinguished either electrophoretically or ultracentrifugally from lipovitellin in sodium chloride solutions. In slightly alkaline solvents (pH 9.0) of low ionic strength ($\mu = 0.3$) the presence of a component similar to γ-livetin could be demonstrated by both sedimentation and electrophoretic procedures in whole egg yolk and to the extent of about 25% in the S1 fraction as originally prepared (5). It seems likely that all samples of lipovitellin prepared by dilution precipitation from sodium chloride (1, 2, 3) have contained γ-livetin as an impurity.

The high phosphorus content of lipovitellin has been attributed to the presence of the phosphorus-rich protein, phosvitin, first isolated by Mecham and Olcott (6). Their procedure did not establish whether phosvitin is an integral part of the lipovitellin molecule (7) or two separate entities that interact strongly. Electrophoretic separation has been claimed (8) but this component has not been evident ultracentrifugally in sodium chloride or buffer solutions. A procedure that would permit the separation and recovery of both lipovitellin and phosvitin in a form suitable for characterization would

[1]Manuscript received December 2, 1957.
Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa, Canada.
Issued as N.R.C. No. 4628.
[2]Postdoctorate Fellow, 1956–57. Present address: National Chemical Research Laboratory, Council for Scientific and Industrial Research, Pretoria, South Africa.

be necessary to establish their existence as separate entities. The present paper describes resolution of the S1* fraction containing lipovitellin, phosvitin, and γ-livetin, and characterizes the lipovitellin component. The properties of purified phosvitin are described in another paper (9).

Methods

Phosphorus was determined by the method of King (10) and total lipid by the procedure previously described (4).

The concentration of dissolved solids in solutions or dialyzates was determined by drying aliquots to constant weight at 60° C. in vacuum. Specific refractive increments (dn/dc) were measured in a differential refractometer calibrated by dry weights as determined above. The refractive index increment was used for the routine determination of concentration.

Most of the physical methods have been described in earlier papers. A MacInnes float apparatus (11, 12) was used to obtain specific volumes at 25° C. Diffusion coefficients were determined at 20° C. with an integral fringe diffusion apparatus constructed from Hilger schlieren optical equipment modified to give a Rayleigh system of the type used by Longsworth (13).

During the preparative steps the proportion of the several components was determined from schlieren patterns obtained ultracentrifugally. The original plates were magnified eight times (linear), traced, and the areas determined with a mechanical integrator. The proportions reported for most of the treatments were not corrected for the difference in refractive index increments of the components or the Johnston–Ogston effect (14).

Results

Preparation and Fractionation

The preparative steps were done at 4° C. Yolks were obtained from fresh hen eggs and were freed from adhering white and membranes as described previously (4). As starting materials for the isolation of lipovitellin, either the total yolk contents (S1, S2, and R fractions) or the sedimenting portions (S1 and S2 fractions) were used. The sedimenting material was prepared by diluting the yolk fluids with four volumes of 10% sodium chloride, stirring for 1 hour, and centrifuging for 16 hours at 78,000 × g. These conditions concentrated the R fraction at the surface, permitting the clear subnatant (S1 and S2) to be removed by piercing the bottom of the centrifuge tube.

The sedimenting fraction was examined in several aqueous solvents since the resolution of γ-livetin and S1 ultracentrifugally depends on the solvent used. The results of this examination are summarized in Table I and some typical sedimentation diagrams in Fig. 1. In most solvents three peaks were evident and, based on their sedimentation behavior (1, 4, 15), these have been identified as S1 (lipovitellin–phosvitin complex), γ-livetin, and S2 (unresolved α- + β-livetin).

*In this paper the term S1 refers to the fastest sedimenting component regardless of the proteins it contains.

TABLE I

EXAMINATION OF THE SEDIMENTING FRACTION OF EGG YOLK IN DIFFERENT AQUEOUS SOLVENTS

Solvent	Ionic strength	pH	Proportion of components S1,* %	γ, %	α+β, %
5% NaCl			56	3	41
Barbiturate buffer	1.0	9.0	41	16	43
Barbiturate buffer	0.3	9.0	33	27	40
As above, dialyzed back to 5% NaCl			42	13	45
Barbiturate buffer	0.2	9.0	38	20	42
Glycine buffer	0.3	10.4	30	30	40
Glycine buffer	0.1	10.4	31	28	41
Phosphate buffer	0.3	7.6	35	19	46
As above, dialyzed back to 5% NaCl			42	13	45
0.104 *M* $(NH_4)_2CO_3$			35	26	39
0.136 *M* Na citrate			31	21	48
0.4 *M* $MgSO_4$			56	–	44

*S1 represents the lipovitellin–phosvitin complex.

The results show that the proportion of S2 is constant (*ca.* 42%) in all solvents but the relative amounts of S1 and γ-livetin are strongly dependent on the solvent used. Little or no γ-livetin is evident in sodium chloride or magnesium sulphate solutions but in the barbiturate and glycine buffers γ-livetin and S1 appear in essentially equal proportions. When a solution of the sedimenting fraction in barbiturate buffer was dialyzed exhaustively against 5% sodium chloride, the apparent amount of γ-livetin decreased markedly. Representative results, reported in Table I, indicate that solvents of low ionic strength and high pH favor the separation of γ-livetin from S1.

The barbiturate buffer was preferred to the glycine buffer because of its lower pH. Unless stated otherwise, subsequent examinations were made with a barbiturate buffer of the following composition: 0.03 *M* barbituric acid, 0.05 *M* sodium barbiturate, and 0.25 *M* sodium chloride, to give pH 9.0 and $\mu = 0.3$.

The removal of γ-livetin from the S1 fraction was attempted by three methods of precipitation: by dilution from various solvents; by ammonium sulphate; and by alcohol at low temperatures. Numerous variations were tried, including fractionation by successive additions of precipitant, and repeated precipitations under similar conditions. The composition of the precipitates from certain treatments is given in Table II. All precipitates obtained by the dilution method contained about one-third γ-livetin, showing that the buffers and other solvents were no better than sodium chloride solutions for separating these proteins. In addition, although lipovitellin readily precipitates at pH 4.0, this method was also ineffective and yielded precipitates containing 42% γ-livetin.

Precipitation with saturated ammonium sulphate solution by stepwise addition to the sedimenting fraction in 10% sodium chloride yielded a small initial precipitate of reasonably pure γ-livetin (only 3% S1) but otherwise no significant separation of S1 and γ-livetin was achieved by this procedure.

TABLE II

COMPOSITION OF SOME PRECIPITATES OBTAINED DURING FRACTIONATION EXPERIMENTS

Solute	Solvent	Precipitation conditions and no. of precipitations	Composition of precipitates* S1,† %	γ-livetin, %
		(A) Precipitation by dilution		
Whole yolk	5% NaCl	At 1.6%, 3rd ppt.	75	25
Whole yolk	Buffer (*a*)‡	At μ = 0.075, 4th ppt.	62	38
Sed. fraction	Buffer (*a*)‡	At μ = 0.15, 0.10, or 0.05	68	32
Sed. fraction	10% NaCl	I At 0.62%, 2nd ppt., 4° C.	64	36
Sed. fraction	0.14 *M* Na citrate	At 0.05 *M*, 2nd ppt.	66	34
		(B) Precipitation with ammonium sulphate		
Whole yolk	10% NaCl	I At 35% sat. (yield 18% of total)	3	94
Whole yolk	10% NaCl	II At 45% sat. (yield 32% of total)	55	39
Whole yolk	10% NaCl	III At 50% sat. (yield 39% of total)	54	31
Fraction BII	10% NaCl	At 50% sat. (yield 75% of BII)	71	29
Fraction BII	10% NaCl	At 60% sat. (yield 25% of BII)	67	33
		(C) Precipitation with ethanol		
Whole yolk	Buffer (*b*)‡	At 6.3%, 4th ppt.	82	18
As AI above	Buffer (*b*)‡	I At 4.7%	78	22
As AI above	Buffer (*b*)‡	II At 5.9%	76	24
As AI above	Buffer (*b*)‡	III At 7.7%	70	30
Fraction CI	Buffer (*b*)‡	At 5.4%	88	12
Fraction CII	Buffer (*b*)‡	At 5.6%	88	12

*Figures will not total 100% if precipitates contained S2.
†S1 represents the lipovitellin–phosvitin complex.
‡Buffer (*a*), barbiturate–NaCl buffer, μ=0.3, pH 9. Buffer (*b*) same but with LiCl.

For the low temperature alcohol fractionation a barbiturate – lithium chloride buffer (μ=0.3, pH 9.0) in water and in 50–50 ethanol–water were used as solvent and precipitant respectively. By using precooled alcohol–water mixtures the temperature was kept strictly at $\pm$ 0.5° C. during fractionation. The first precipitate (Table II) contained reasonably pure S1 (88%) but the yields by this method were extremely small and the method of preparation was therefore considered unsuitable.

Although γ-livetin was not resolved ultracentrifugally from the S1 complex in magnesium sulphate solutions (Table I), this solvent appears to be important in effecting the separation of phosvitin from lipovitellin (6). Solutions of whole egg yolk in 0.4 *M* magnesium sulphate were therefore tested for their ability to break up the S1 complex and facilitate the removal of γ-livetin. The composition of the precipitates obtained by stepwise dilution of this solvent appears in Table III. The first precipitate (Fraction I) contained about 73% of a component having a sedimentation coefficient (*S*) *ca.* 2 and phosphorus content similar to that of phosvitin. Fraction II also contained phosvitin and subsequent fractions showed an increasing proportion of γ-livetin. As Fraction II appeared to be the best source of lipovitellin, it

TABLE III

COMPOSITION OF PRECIPITATES OBTAINED BY STEPWISE DILUTION OF WHOLE EGG YOLK IN 0.4 *M* MAGNESIUM SULPHATE

Fraction	Precipitated at:	Proportion of total protein precipitated, %	Composition of precipitates: Initial — Lipovitellin, %	γ-Livetin, %	Phosvitin, %	After further purification* — Lipovitellin, %	γ-Livetin, %
I	0.2 *M* $MgSO_4$	23.9	15	12	73		
II	0.1 *M* $MgSO_4$	10.7	58	16	26	86	11
III	0.05 *M* $MgSO_4$	55.0	70	30	–	73	27
IV	0.025 *M* $MgSO_4$	10.4	44	56	–	58	42
Starting material: Fraction III							
III_1	0.1 *M* $MgSO_4$	28.7	84	16	–	85	15
III_2	0.05 *M* $MgSO_4$	64.4	70	30	–	74	26
III_3	0.025 *M* $MgSO_4$	6.9	49	51	–	49	51

*Initial precipitates were dissolved in barbiturate buffer and precipitated by dilution with three volumes of water.

TABLE IV

SUMMARY OF CHEMICAL AND PHYSICAL MEASUREMENTS ON LIPOVITELLIN

Property	Lipovitellin (Fraction II)	Lipovitellin	Lipovitellin in 4.0 *M* urea
Lipid, %	19.6	20.2	14.9
Phosphorus (lipid-free basis), %	0.78	0.49	
$dn/dc \times 10^3$, g./dl., at λ = 5780 Å	1.76 ± 0.01	1.81 ± 0.01	1.98 ± 0.01
$\bar{v}$, ml./g.	0.774	0.781	0.737
$S^0_{20,w}$, Svedbergs			
Buffer (*a*)	10.8	11.1	
Buffer (*b*)		10.9	
Buffer (*c*)			6.0
ds/dc, Sved./1%			
Buffer (*a*)	−0.58	−0.81	
Buffer (*b*)		−0.90	
Buffer (*c*)			−0.30
$D_{20,w} \times 10^7$, $cm.^2$ $sec.^{-1}$			
Buffer (*a*), lipovitellin conc. = 0.28%		3.81	
Buffer (*b*), lipovitellin conc. = 0.25%		3.90	
Molecular weight		3.15×10^5	
Frictional ratio		1.2	

NOTE: Buffer (*a*), barbiturate, μ=0.3, pH 9.0.
Buffer (*b*), 1.0 *M* NaCl.
Buffer (*c*), barbiturate (μ = 0.3, pH 9.0) + 4.0 *M* urea.

was dissolved in barbiturate buffer and precipitated by dilution with three volumes of water. This procedure yielded an S1 fraction containing 86% lipovitellin. A few physical and chemical measurements made on this material appear in Table IV. The presence of some phosvitin is indicated by the phosphorus content of 0.78% on a lipid-free basis.

During these studies it was observed (9) that phosvitin forms a high molecular weight complex (S=62) in the presence of magnesium ions. Consequently a preparation corresponding to the supernatant from Fraction I

(Table III) was adjusted by dialysis to 0.05 *M* magnesium sulphate in barbiturate buffer. The analytical centrifuge revealed that 15% of the material was present as a component with $S=62$. By centrifuging the solution at 105,000 g in a preparative machine for 1 hour, decanting off the supernatant, and repeating the procedure, this heavy component was removed as indicated by the analytical ultracentrifuge.

The following method was then devised for the preparation of lipovitellin, all steps being performed at 4° C.: to 400 g. egg yolk, 1200 ml. 0.4 *M* $MgSO_4$ and 39.4 g. solid $MgSO_4.7H_2O$ were added. This mixture was stirred for 1 hour, centrifuged for 30 minutes at 2500 r.p.m., and the floating lipid material removed by decantation and filtration. This filtrate was slowly diluted with an equal volume of water and the precipitate (mainly phosvitin) removed by centrifugation.

The supernatant solution was again diluted with an equal volume of cold water. The precipitate (mainly lipovitellin) was removed, dissolved in 500 ml. barbiturate buffer, and precipitated by the addition of three volumes of cold water. This precipitate was redissolved in 50 ml. of 10% sodium chloride and freed from low density lipoproteins and free fat by centrifugation at 105,000 *g* for 2 hours. The clear solution was carefully removed and dialyzed for at least 16 hours against barbiturate buffer containing 0.05 *M* magnesium sulphate. This solution was centrifuged at 105,000 *g* until the supernatant was completely free from the 62 *S* phosvitin complex.

Physical Measurements

The electrophoretic and sedimentation diagrams of one of the lipovitellin preparations appear in Fig. 2. The electrophoretic analysis (Fig. 2,A and B) shows the presence of a small amount of impurity of higher mobility, while the ultracentrifuge (Fig. 2C) shows about 3–4% of a faster, and 11% of a slower, sedimenting component. The slowest sedimenting material occupies the same position as γ-livetin. The nature of the fastest sedimenting impurity is not known: it moves more slowly than the magnesium–phosvitin complex but it may be a complex of phosvitin with other material.

The results of the physical and chemical measurements on lipovitellin are summarized in Table IV. It must be recognized that these samples still contained about 10% γ-livetin and possibly some phosvitin, as the phosphorus content was 0.49% on a lipid-free basis. Until the phosphorus content of

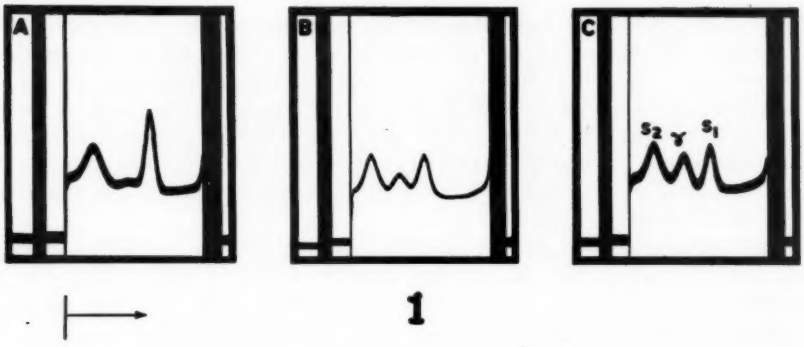
---

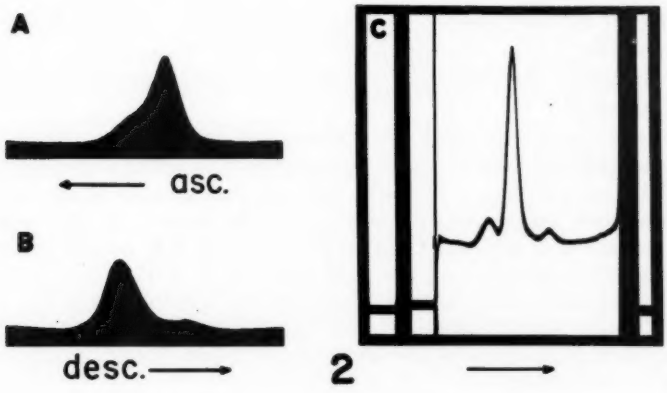
FIG. 1. Sedimentation diagrams of sedimenting fraction.
A. In 5% NaCl.
B. In phosphate buffer, $\mu=0.3$, pH 7.6.
C. In barbiturate buffer, $\mu=0.3$, pH 9.0.

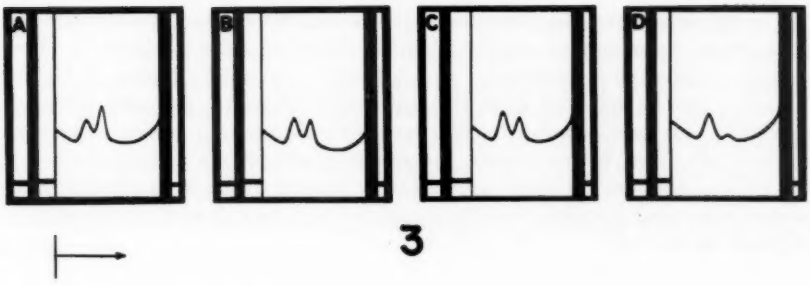
FIG. 2. Electrophoretic (A, B) and sedimentation (C) diagrams of lipovitellin prepared as described, all in barbiturate buffer.

FIG. 3. Sedimentation diagrams of lipovitellin in 2 *M* and 4 *M* urea.
A. In 2 *M* urea, 16 hours.
B. In 2 *M* urea, 44 hours.
C. In 2 *M* urea, 64 hours.
D. In 4 *M* urea, 17 hours.

pure vitellin is known, it is impossible to establish the extent of the phosvitin impurity. As compared with earlier measurements (1) on the S1 fraction (containing all the phosvitin and about 25% γ-livetin), the partial specific volume and sedimentation coefficient of the present material are both higher. Together with the diffusion coefficient, they yield a molecular weight of 3.2×10^5.







It was found impossible to confirm this molecular weight by other methods. The molecule is obviously too large for satisfactory osmotic pressure measurements. Likewise, filtration for light scattering always resulted in the appearance of fibrous material in the filtrate, presumably arising from changes induced during filtration.

The molecular weight reported here for lipovitellin appears to be the best available and subject to only minor corrections for its comparatively low γ-livetin content. Using this value and the molecular weight of γ-livetin (15) it appears that the previously reported (1) value (3.3 to 4.0 $\times 10^5$) for the S1 fraction is too low if these two proteins combine, as suggested by their sedimentation behavior in sodium chloride solutions, and too high, if they behave as independent entities. The properties of the S1 fraction were therefore re-examined by isolating (repeated precipitation at 0.62% sodium chloride) and examining this material in 10% sodium chloride solution by the methods used in the present study.

This preparation showed only one component in the ultracentrifuge having the following properties: $dn/dc = 1.80 \times 10^{-3}$; $\bar{v} = 0.771$ ml./g.; $S^{\circ}_{20,w} = 10.3$ Svedbergs; $D_{20,w} = 3.13 \times 10^{-7}$ cm.^{2} sec.$^{-1}$, and $M_w = 3.5 \times 10^5$. The specific volume is somewhat higher than previously reported and may be due to a higher lipid content which was not determined. The results of the diffusion determination yielded a linear $(\Delta H/nf)^2$ vs. time plot (13) but some instability was indicated by the presence of a small amount of a faster sedimenting component after 3 days' exposure to 20° C. in the diffusion cell. In spite of these uncertainties the molecular weight was in good agreement with those reported earlier (1).

In a further attempt to break the complex and obtain lipovitellin of higher purity, a sample prepared as above was examined in barbiturate buffer containing 2 *M* and 4 *M* urea. The sedimentation diagrams are shown in Fig. 3. In 2 *M* urea two components are evident and the slower one increases with time, in contact with urea (Fig. 3,A, B, and C). In 4 *M* urea lipovitellin is almost entirely converted into the slow component in 17 hours (Fig. 3D). This slow component with $S = 6.0$ is suggestive of a splitting of the lipovitellin molecule. Precipitation occurred when the urea was removed by dialysis, indicating an irreversible change. This subject is still under investigation.

Discussion

The major sedimenting component (S1) of egg yolk, which yields only one peak when examined in sodium chloride solutions, is a complex of three proteins—lipovitellin, phosvitin, and γ-livetin. In dilute buffers (pH 9.0) γ-livetin can be resolved from the lipovitellin–phosvitin complex ultracentrifugally, but precipitation by dilution, with ammonium sulphate, or with ethanol, all failed to fractionate the material adequately. A tendency for the first ammonium sulphate precipitate to have the highest content of lipoprotein somewhat parallels the behavior of the γ-globulin and the β-lipoprotein of blood serum (16).

Magnesium sulphate solutions proved to be the best solvents for fractionation. This salt apparently acts on the lipovitellin–phosvitin complex and enables a useful separation of all three components to be accomplished on subsequent precipitation by solvent dilution, although lipovitellin so prepared still contained minor amounts of γ-livetin. In the presence of low concentrations of magnesium ions, phosvitin forms a soluble, high molecular weight complex that can be removed from the lipovitellin solutions by centrifugation. While this is a useful purification step, there is no direct evidence that it removes all the phosvitin.

The present results show that the three proteins of the S1 complex can be fractionated to a considerable extent without apparent damage to any of the entities. Since a molecular cleavage yielding phosvitin as a fragment would be expected to cause a gross change in the properties of the residue, phosvitin is evidently a separate protein and not an integral part of the lipovitellin molecule.

Considering the strong interactions between the three proteins of the S1 fraction, as indicated by the difficulties in their separation, it would appear that the reported proportion of lipovitellin in egg yolk (7, 17) actually refers to some form of this complex rather than to lipovitellin. Current information (7, 17) indicates that lipovitellin and phosvitin represent over three-quarters, and the livetins only a quarter, of the sedimenting proteins. Although the Johnston–Ogston effect (14) may act to enhance the apparent amount of the slower sedimenting livetins, this effect could not account for the results under the conditions of the present sedimentation analysis. Evidently about two-thirds of the sedimenting material are the livetins and only about one-third the lipovitellin–phosvitin complex. The reported composition (7, 17) of lipovitellin with respect to phosphorus, sulphur, and amino acids may likewise have little significance, since the method of preparation would yield material containing appreciable amounts of phosvitin and γ-livetin.

Until pure lipovitellin is available, little can be said regarding its composition. Reducing the γ-livetin content from about 25 to 10% and removing part, if not all, of the phosvitin increased the lipid content from 18.2 (1) to 20.2%. Correction for the γ-livetin impurity would raise it to about 22% but the constancy of the lipid content has yet to be established. The fractionation method described here reduced the protein phosphorus content of lipovitellin to about half that previously reported (7). If vitellin is not a phosphoprotein, this could indicate the presence of about 5% phosvitin as an impurity. It seems probable, however, that vitellin contains some phosphorus.

The specific volume ($\bar{v}$) and sedimentation coefficient of the present preparation were both higher than that previously observed for the S1 fraction in sodium chloride solutions (1). These values are not strictly comparable since they were observed in different solvents but since both γ-livetin (15) and phosvitin (9) have a lower $\bar{v}$ than lipovitellin, and were present in greater proportion in the S1 fraction, the present value is consistent with the earlier finding.

Using the methods employed in this study the S1 fraction prepared and examined in sodium chloride solution was found to have a molecular weight of 3.5×10^5. This and the earlier results (1) are adequate to show that the size of the S1 fraction does not approach the sum of the molecular weights of γ-livetin and lipovitellin, although these proteins are present in about equal molecular proportions (Table II) and do not separate in sodium chloride solutions. Conversely a still lower mean molecular weight would have been expected had these two proteins behaved as independent entities. Evidently the S1 fraction does not behave as a single kinetic unit in sodium chloride solutions, but the rate and extent of interaction between the proteins in this fraction are sufficient to prevent their resolution by sedimentation. The instability and anomalous sedimentation properties of γ-livetin (15) may also have contributed to the observed behavior.

The behavior of lipovitellin in urea–buffer solutions suggests, first, that the lipovitellin molecule consists of two parts held together by secondary valence forces, and second, that the two parts are sufficiently similar in size to remain unresolved during ultracentrifugation. The degradation products were not resolved by sedimentation from the residual γ-livetin impurity, but a combination of lipid loss, changes in molecular shape, or interaction, could explain this behavior.

The older methods of preparing lipovitellin, and the phosphorus content of the preparations (7, 17) indicate that it was a mixture of three proteins, two of which were not lipoproteins. The term "lipovitellin" should now be reserved strictly for the lipoprotein component, and material prepared by the older methods designated "lipovitellin complex".

Acknowledgments

The authors wish to thank Mr. D. R. Muirhead for technical assistance and the Poultry Division, Canada Department of Agriculture, for supplying fresh eggs as required.

References

1. VANDEGAER, J. E., REICHMANN, M. E., and COOK, W. H. Arch. Biochem. Biophys. **62**, 328 (1956).
2. CHARGAFF, E. J. Biol. Chem. **142**, 491 (1942).
3. ALDERTON, G. and FEVOLD, H. L. Arch. Biochem. **8**, 415 (1945).
4. MARTIN, W. G., VANDEGAER, J. E., and COOK, W. H. Can. J. Biochem. and Physiol. **35**, 241 (1957).
5. MARTIN, W. G. and COOK, W. H. Unpublished results.
6. MECHAM, D. K. and OLCOTT, H. S. J. Am. Chem. Soc. **71**, 3670 (1950).
7. WARNER, R. C. The proteins. Vol. II. Pt. A. Academic Press, Inc., New York. 1954. p. 435.
8. SUGANO, H. J. Biochem. Tokyo, **44**, 205 (1957).
9. JOUBERT, F. J. and COOK, W. H. Can. J. Biochem. and Physiol. **36**, 399 (1958).
10. KING, E. J. Biochem. J. **26**, 294 (1932).
11. MACINNES, D. A., DAYHOFF, M. O., and RAY, B. R. Rev. Sci. Instr. **22**, 642 (1951).
12. CHARLWOOD, P. A. J. Am. Chem. Soc. **79**, 776 (1957).
13. LONGSWORTH, L. G. J. Am. Chem. Soc. **74**, 4155 (1952).
14. JOHNSTON, J. P. and OGSTON, A. G. Trans. Faraday Soc. **42**, 789 (1946).
15. MARTIN, W. G. and COOK, W. H. Can. J. Biochem. and Physiol. **36**, 153 (1958).
16. EDSALL, J. T. Advances in Protein Chem. **3**, 383 (1947).
17. FEVOLD, H. L. Advances in Protein Chem. **6**, 187 (1951).

PREPARATION AND CHARACTERIZATION OF PHOSVITIN FROM HEN EGG YOLK[1]

F. J. JOUBERT[2] AND W. H. COOK

Abstract

Phosvitin was precipitated from egg yolk solution in 0.4 *M* magnesium sulphate by adding an equal volume of water. Purification was accomplished by repeatedly precipitating and dialyzing at pH 4.0 to remove lipovitellin. Phosvitin so prepared was free of lipid, contained 9.6% phosphorus, and had a molecular weight of 3.0×10^4 at pH 4.0 and a frictional ratio that indicates an elongated molecule. It is a polyelectrolyte with properties that are dependent on both the concentration of the solute and the composition of the solvent. In buffer solutions containing low concentrations (0.01 to 0.05 *M*) of magnesium sulphate, it forms complexes having a particle weight of 14×10^6 and with calcium salts a particle weight of 7.5×10^6.

Introduction

The yolk of hen egg contains about 0.6% phosphorus (1) in the form of both phospholipids and phosphoproteins. The older methods of fractionating the yolk constituents indicated that the lipovitellin fraction contained most of the phosphoproteins, although the phosphorus content showed considerable variation (1). Later, Mecham and Olcott (2) isolated a protein, which they termed phosvitin, containing 9.7% phosphorus, that accounted for most of the protein phosphorus in egg yolk, but lipovitellin was not recovered from this preparation. This raised the question as to whether phosvitin was a degradation product of lipovitellin or a separate protein that interacts strongly with lipovitellin (3). Recently the authors (4) have fractionated egg yolk and obtained both lipovitellin and phosvitin, thereby establishing that these proteins exist as separate entities. A simplified method of preparing phosvitin and its characterization with respect to certain physical properties, including its molecular weight, are reported in this paper.

Experimental

Methods

Details of the methods have been described in earlier papers (1, 4, 5). Phosphorus was determined by the method of King (6) and nitrogen by micro-Kjeldahl.

Preparation

The preparations were made at 4° C. The procedure used was based on Mecham and Olcott's (2) observation that phosvitin could be precipitated in the presence of the appropriate concentration of magnesium sulphate.

[1]Manuscript received December 2, 1957.
Contribution from Division of Applied Biology, National Research Laboratories, Ottawa, Canada.
Issued as N.R.C. No. 4626.
[2]Postdoctorate Fellow, 1956–57. Present address: National Chemical Research Laboratory, Council for Scientific and Industrial Research, Pretoria, South Africa.

As shown in a previous paper (4) a dispersion of egg yolk in three volumes of 0.4 *M* magnesium sulphate, when diluted with an equal volume of water, gives a precipitate containing about 73% phosvitin, 12% γ-livetin, and 15% lipovitellin.

The precipitate so obtained from 400 g. of egg yolk was redissolved in 800 ml. of 0.4 *M* magnesium sulphate and reprecipitated. This precipitate was dissolved in 100 ml. of 10% sodium chloride and centrifuged for 2 hours at 105,000 *g* to float any lipid material. The subnatant was carefully removed, diluted with 200 ml. of water, and dialyzed against several changes of acetate buffer ($\mu = 0.1$, pH 4.0) to precipitate the remaining lipovitellin. The precipitate was removed by centrifuging and the supernatant solution was dialyzed free from solids and freeze-dried. This material was found to have the chemical and physical properties of phosvitin (2).

Several modifications of this basic procedure were tested. Little additional phosvitin was obtained by further dilution from 0.2 *M* to 0.1 *M* magnesium sulphate (4). According to Mecham and Olcott (2) dialysis against 0.4 *M* citrate buffer at pH 5.0 removes appreciable amounts of heavy metal impurities. Such dialysis had no effect on the sedimentation coefficients (Table I) and was found unnecessary in the method described.

Properties of Phosvitin

The results of the physical measurements on phosvitin are summarized in Table I. The preparations contained 9.6% phosphorus, 12.6% nitrogen, and no lipid. The same values were obtained on several preparations and, except for the somewhat higher nitrogen content, are the same as reported by Mecham and Olcott (2).

TABLE I

SUMMARY OF THE PHYSICAL MEASUREMENTS OF PHOSVITIN

Property	Value observed	Solvent used
$dn/dc \times 10^3$ at $\lambda = 5780$ Å, g./dl.	1.64 ± 0.02	0.1 *M* NaCl
$\bar{v}$, ml./g.	0.545 ± 0.005	0.1 *M* NaCl
Mobility × 10^5, cm.^{2} sec.$^{-1}$ volt^{-1}		
pH = 4.0	−7.3 (desc.), − 9.2 (asc.)	Acetate, μ = 0.1
pH = 9.0	−7.5 (desc.), −10.7 (asc.)	Barbiturate, μ = 0.2

Solvent	0.5 *M* NaCl	Acetate buffer*	Barbiturate buffer†	Phosphate buffer‡
$S^0_{20,w}$, Svedbergs	3.14	3.14 (Prep. A)	3.25	2.92
(Prep. No. in parentheses)	3.08§	3.17 (Prep. B)		
$D_{20,w} \times 10^7$, cm.^{2} sec.$^{-1}$	5.43 (0.23%)	5.43 (0.23%)	5.42 (0.51%)	5.79 (0.54%)
(concn. given in parentheses)	5.22 (0.42%)	5.12 (0.43%)		
		5.06 (0.44%)		
Molecular weight × 10^{-4}	3.09	3.04	3.20	2.69
Frictional ratio	2.08	2.09	1.98	2.04

$S^0_{20,w}$ in 0.1 *M* citrate, pH = 5.0, = 2.72 Svedbergs.
*μ = 0.1, pH = 4.0.
†μ = 0.1, pH = 9.0.
‡μ = 0.1, pH = 7.0.
§Citrate dialyzed preparation in 0.1 *M* NaCl.

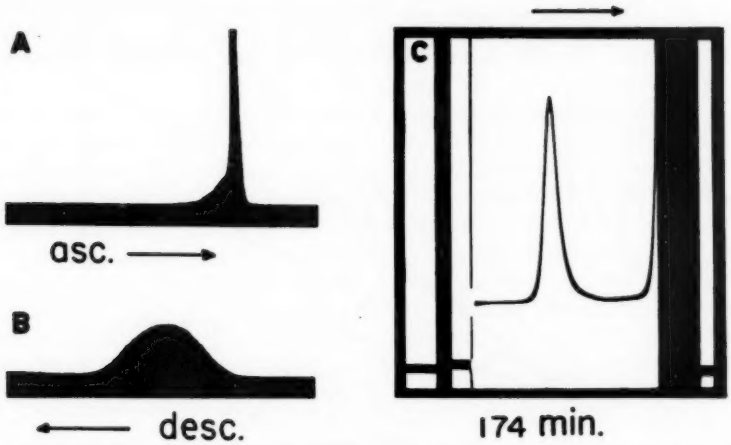


FIG. 1. Electrophoretic (A, B) and sedimentation (C) diagrams of phosvitin prepared as described, all in acetate buffer, $\mu = 0.1$, pH = 4.0.

The electrophoretic and sedimentation patterns in acetate buffer ($\mu = 0.1$, pH 4.0) both showed only a single component (Fig. 1). Although the electrophoretic patterns of the ascending and descending boundaries show differences in shape and mobility, the observed mobilities (Table I) at pH 4.0 and 9.0 were quite similar. This behavior, together with the titration curve (2) shows that phosvitin is a negatively charged polyelectrolyte throughout this pH range.

The specific volume $\bar{v}$ of 0.545 was determined in 0.1 M sodium chloride. This low value must be attributed to the acidic phosphate groups and the compensating gegenions. Although the effect of the gegenion on $\bar{v}$ may be affected by the pH of the solution, the above value was used with all solvents. This will be discussed later in relation to the other measurements.

The sedimentation coefficients of phosvitin were concentration dependent, requiring $1/S$ vs. C plots for extrapolation to obtain $S^{\circ}_{20,w}$ (Fig. 2A). Since phosvitin is a polyelectrolyte, the ionic strength of the solvent must be high enough to swamp charge effects during sedimentation. The ionic strength required was determined at fixed protein concentration in 0.05, 0.10, 0.15, and 0.20 M sodium chloride solutions: the values obtained were 2.55, 2.68, 2.72, and 2.76 Svedbergs, respectively. The sedimentation behavior of phosvitin at various concentrations in several solvents is shown in Fig. 2A. Clearly, the residual charge has little effect on the sedimentation rate above $\mu = 0.1$ (the lowest used) and its influence is reduced to within experimental error, along with the true concentration dependence, by extrapolation to zero concentration.

The observed $S^{\circ}_{20,w}$ varied from a minimum of 2.72 S in 0.1 M citrate buffer at pH 5.0, to 3.25 S in barbiturate buffer at pH 9.0 (Table I). This variation is evidently not due to differences between preparations since the

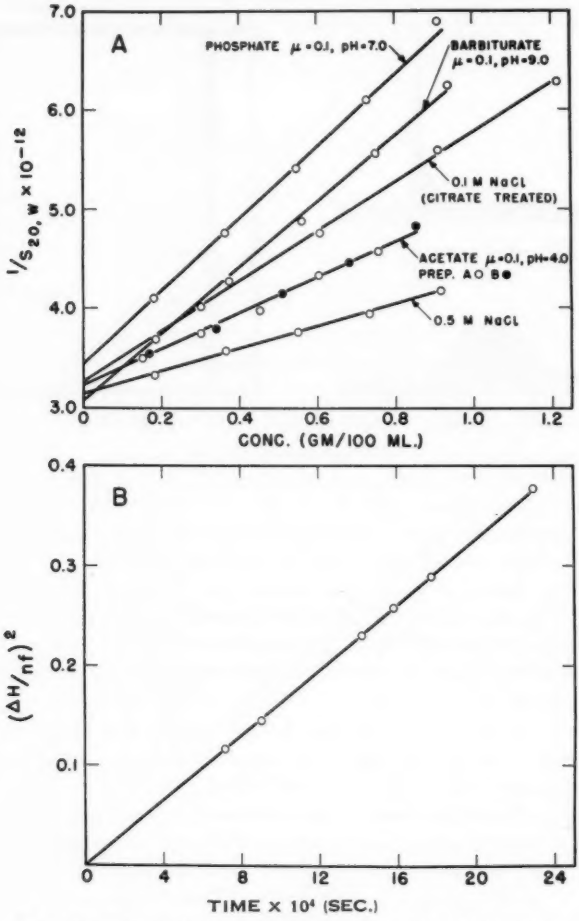


FIG. 2. Sedimentation and diffusion properties of phosvitin.
A. $1/S$ vs. C plots in various solvents.
B. $(\Delta H/nf)^2$ vs. time plot in acetate, $\mu = 0.1$, pH = 4.0.

sedimentation coefficients of different preparations agreed within experimental error (e.g. Preparations A and B, Table I). Although S might be expected to increase with pH from the uptake of gegenions, this fails to explain the results in acetate buffer at pH 4.0, where a value of 3.15 S was observed. The sedimentation coefficients in acetate and barbiturate buffers, and in sodium chloride solutions, covering the pH range from 4.0 to 9.0, differed by only 5% between these extremes and suggest that the effect of pH is comparatively small. The lower sedimentation coefficients observed in sodium citrate solutions and phosphate buffers must therefore be attributed to some specific effect of these ions.

Results representative of the diffusion measurements are reported in Fig. 2B and Table I. The plot of $(\Delta H/nf)^2$ vs. time (7) was linear for all measurements, showing that the diffusion rate of phosvitin does not change over the 3-day period at 20° C. Duplicate determinations indicated an experimental error of about 2% at the lower protein concentrations but somewhat higher over the entire concentration range. The diffusion coefficients tended to decrease rather than increase with increasing phosvitin concentration but this departure from the usual behavior was not established with certainty since the slope did not exceed the confidence limits calculated from the overall experimental error. In consequence the values observed at the lowest phosvitin concentration used in each solvent (Table I), rather than the extrapolated or average values, were used to calculate the molecular weights. In sodium chloride and acetate buffer solutions, the solvents studied most extensively, the diffusion coefficients were 5.43×10^{-7} at phosvitin concentrations of 0.23%, and these would have been about 5% higher had extrapolation to zero concentration been considered valid. The highest value observed was in phosphate buffer (5.79×10^{-7}) and although this involved only one protein concentration, it is suggestive of a specific ion effect.

Using the sedimentation and diffusion coefficients appropriate to each solvent, the molecular weight in acetate buffer, sodium chloride solutions, and barbiturate buffer was 3.04, 3.09, and 3.20×10^4, respectively. This slight increase with pH is in accordance with expectation and its significance will be discussed later. In phosphate buffer, where the sedimentation coefficient was lowest and the diffusion coefficient highest, the molecular weight was 2.69×10^4.

The frictional ratio derived from these values varied from 1.98 to 2.09 in the four solvents, indicating that phosvitin is an elongated molecule. If it is assumed that these frictional effects arise entirely from elongation and that the molecule can be represented by a prolate ellipsoid, the axial ratio lies between 20 and 25. Since some solvation is bound to occur, this must represent a maximum extension.

Properties of Complexes of Phosvitin with Metallic Cations

The method of preparation and the results of the physical measurements indicate that phosvitin is strongly influenced by interactions with the solvent. The precipitation of phosvitin by magnesium sulphate was therefore examined in different ionic environments. In these experiments aliquots of a 1% phosvitin solution in water were dialyzed against the different buffers containing various amounts of magnesium sulphate and then examined in the ultracentrifuge. When precipitation occurred the precipitate was centrifuged off and the amount of protein in the supernatant determined.

The results, summarized in Table II, show that at low magnesium sulphate concentrations no change could be detected (see also Table III); at intermediate concentrations extremely high molecular weight complexes are revealed in the ultracentrifuge while still higher concentrations cause pre-

TABLE II

EXAMINATION OF PHOSVITIN IN BUFFERS CONTAINING MAGNESIUM SULPHATE

Buffer	μ	pH	$MgSO_4$ concn., M	Protein ppt., % of total	Protein as fast comp., % of total	$S_{20,w}$, Svedbergs for fast comp.
0.1 M NaCl			0.04	Nil	Nil	–
Acetate	0.1	4.0	0.01	Nil	Nil	–
	0.1	4.0	0.03	Nil	14	58.0
	0.1	4.0	0.05	Nil	25	60.4
	0.1	4.0	0.07	12	88	–
Acetate	0.1	5.5	0.03	Nil	11	39.6
Barbiturate	0.1	7.0	0.01	Nil	100	56.3
	0.1	7.0	0.02	94	6	–
Barbiturate	0.1	9.0	0.01	Nil	100	52.3
	0.1	9.0	0.03	98	2	–
	0.3	9.0	0.01	Nil	100	32.8
	0.3	9.0	0.03	14	86	–

cipitation. In these and subsequent experiments there was no evidence of the dimer mentioned by Mecham and Olcott (2).

Although the concentration of the bivalent cations is the primary factor affecting complex formation and precipitation, the pH and other ions in the buffer modify the amount required. In barbiturate buffer at pH 9.0 and $\mu = 0.1$, the magnesium sulphate concentration required for complex formation or precipitation is lower than in acetate buffer at pH 4.0, $\mu = 0.1$. This might be expected since the uptake of gegenions would increase with pH. High ionic strengths (barbiturate buffer plus sodium chloride) increase the concentration of magnesium sulphate required. The size of the particles, as judged from the sedimentation coefficients, is also sensitive to the ionic environment.

A physical examination of these phosvitin complexes was undertaken using three solvents: A in 0.1 M sodium chloride plus 0.04 M magnesium sulphate; B in acetate buffer ($\mu = 0.1$, pH 5.5) plus 0.01 M calcium chloride; and C in barbiturate buffer ($\mu = 0.1$, pH 9.0) plus 0.01 M magnesium sulphate. Solvent A was used in an attempt to promote dimer formation (2) but the physical constants (Table III) differed only slightly from those of

TABLE III

SUMMARY OF PHYSICAL MEASUREMENTS ON PHOSVITIN IN THE PRESENCE OF MAGNESIUM SULPHATE AND CALCIUM CHLORIDE

Property	Phosvitin in solvent A*	Calcium–phosvitin complex in solvent B	Magnesium–phosvitin complex in solvent C
$dn/dc \times 10^3$ at $\lambda = 5780$ Å, g./dl.		1.79 ± 0.02	1.99 ± 0.02
$\bar{v}$, ml./g.	0.545 (from Table I)	0.475 ± 0.002	0.403 ± 0.001
$S^0_{20,w}$, Svedbergs	3.58	38.8	62.5
$D_{20,w} \times 10^7$, cm.^{2} sec.$^{-1}$ (concn. of complex in parentheses)	5.60 (0.23%)	2.38 (0.18%) 2.35 (0.34%)	1.71 (0.13%) 1.75 (0.23%) 1.83 (0.36%)
Molecular weight			
From S and D	3.41×10^4	7.54×10^5	14.48×10^5
From light scattering			15.9×10^5
Frictional ratio (f/f_0)	1.95	1.72	1.97

*Solvent A: 0.1 M NaCl + 0.04 M $MgSO_4$.
B: Acetate ($\mu = 0.1$, pH = 5.5) + 0.01 M $CaCl_2$.
C: Barbiturate ($\mu = 0.1$, pH = 9) + 0.01 M $MgSO_4$.

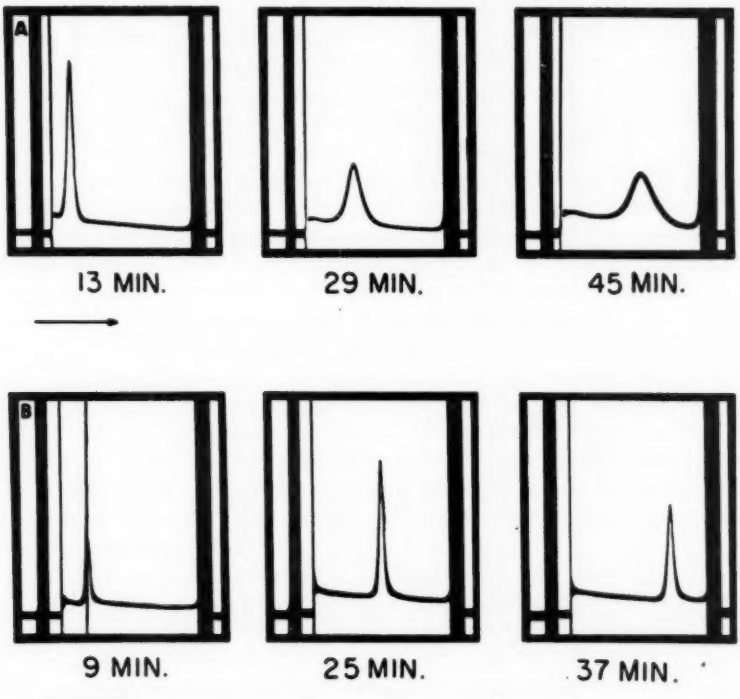


FIG. 3. Sedimentation diagrams of phosvitin complexes.
A. Calcium–phosvitin complex.
B. Magnesium–phosvitin complex.

phosvitin containing no magnesium sulphate. The sedimentation diagrams of phosvitin in solvents B and C (Fig. 3) indicate that these complexes are quite homogeneous. They also have a strong concentration dependence as shown in the $1/S$ vs. C plots in Fig. 4A. Their stability is indicated by the linear relation between $(\Delta H/nf)^2$ vs. time (Fig. 4B) over the 3-day period at 20° C. required for the diffusion measurements.

The results, summarized in Table III, show that the calcium–phosvitin complex (solvent B) had a sedimentation coefficient nearly 10 times as great as the native phosvitin, a lower diffusion coefficient, and a particle weight of 7.5×10^5. The magnesium–phosvitin complex (solvent C) had a still higher sedimentation coefficient, a lower diffusion coefficient, and a particle weight of 14.5×10^5. This last value was confirmed by light scattering which yielded a particle weight of 15.9×10^5.

The frictional ratio of the magnesium–phosvitin is essentially the same as that of native phosvitin, i.e., equivalent to an axial ratio of 20:1 on an anhydrous basis. The frictional ratio for the calcium–phosvitin complex is

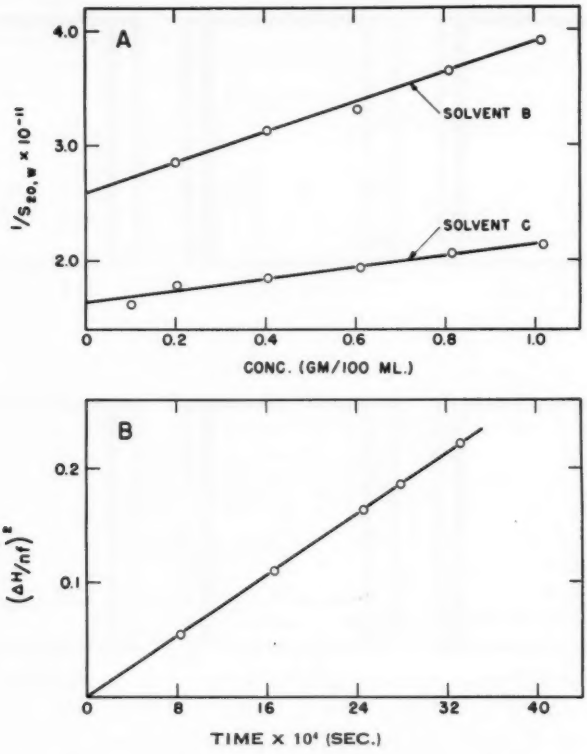


FIG. 4. Sedimentation and diffusion properties of phosvitin complexes.
A. $1/S$ vs. C plots for solvent B (Ca^{++}) and solvent C (Mg^{++}).
B. $(\Delta H/nf)^2$ vs. time plot for magnesium–phosvitin complex.

somewhat lower, the corresponding axial ratio being about 14:1. Again, these represent the maximum extension since hydration doubtless contributes something to the frictional effects.

Discussion

Egg yolk dissolved in 0.4 *M* magnesium sulphate can be fractionated by solvent dilution into phosvitin and lipovitellin. Not only is this method somewhat simpler than the procedures described by Mecham and Olcott (2) but it also establishes that phosvitin is not an integral part of the lipovitellin molecule, since both entities can be recovered.

When sodium citrate or phosphate buffers were used as solvents the sedimentation coefficients were lower than in the other solvents and must be attributed to specific effects of these ions. In the other three solvents (acetate, sodium chloride, and barbiturate) the molecular weights increased from

3.04 to 3.20 $\times$ 10^4 with an increase in pH from 4.0 to 9.0. Phosvitin solutions dialyzed exhaustively against water had a pH of 4.0 and a sodium content of 1.6% (flame photometer) in reasonable agreement with the expected sodium content indicated by titration curves (2). The formation of the sodium salt at higher pH values would increase the apparent molecular weight, and presumably reduce the partial specific volume.

The molecular weight of phosvitin at pH 4.0 was 3.04 $\times$ 10^4, and this value should represent the hydrodynamic entity of lowest sodium content. This molecular weight and a phosphorus content of 9.6% indicate that phosvitin contains about 94 atoms of phosphorus per molecule. The addition of one equivalent of sodium per atom of phosphorus, as indicated by the titration curves (2), would increase the molecular weight to 3.25 $\times$ 10^4, a value in good agreement with that observed at pH 9.0.

The specific volume used to compute the molecular weights was measured on sodium chloride solutions (pH *ca.* 6.8). An inverse relation would be expected between specific volume and pH or the sodium content of phosvitin, and tend to reduce the reported difference between the molecular weights at pH 4.0 and 9.0. Since the molecular weights are not particularly sensitive to small changes in $\bar{v}$ when this value is low, such effects are judged to be within experimental error.

The observed molecular weight of phosvitin at pH 4.0 (3.0 $\times$ 10^4) is higher than that obtained by Mecham and Olcott (1.8 to 2.5 $\times$ 10^4) by osmotic pressure method at higher pH values. Since phosvitin is a polyelectrolyte, osmotic procedures are likely to yield results that are too low. Attempts to confirm these osmotic pressure measurements were unsuccessful. In both acetate buffer and 0.5 *M* sodium chloride solutions the osmotic pressure increased slowly with time and equilibrium was not established after 1 month at 4° C.

The frictional ratio indicates an axial ratio of 20:1 assuming no solvation, and although this would be reduced by frictional effects arising from hydration, phosvitin is evidently an elongated molecule. Using the maximum axial ratio, the dimensions of the molecule are about 14 Å by 280 Å.

Divalent cations might be expected to react with phosvitin and by cross-linkage eventually cause precipitation. It was observed, however, that under a given set of experimental conditions phosvitin forms reasonably monodisperse soluble complexes with calcium and magnesium salts before precipitation occurs. The composition and pH of the solvent as well as the concentration of the divalent cation determine the formation and size of the particles but those observed range from 20 to over 40 phosvitin molecules. Low concentrations of divalent cations cause little complex formation in certain solvents, but, in general, complexes are formed at about 0.01 *M* magnesium sulphate, precipitation occurs at 0.1 *M*, and the precipitate redissolves at 0.4 *M*. Further work is required to characterize these complexes adequately, but the decrease in partial specific volume (Table III) clearly indicates the uptake of calcium and magnesium. Calculations based

on these partial specific volumes (8) suggest a Ca:P ratio of about 1:2 at pH 5.5 (solvent B) and a Mg:P ratio of about 1:1 at pH 9.0 (solvent C).

The observed properties of phosvitin indicate that it should react strongly with other proteins of opposite charge. Obviously it interacts strongly with lipovitellin and although the isoelectric point reported for this protein is pH 5.6 (9), this would seem to be the value applicable to the lipovitellin–phosvitin complex; the isoelectric point of pure lipovitellin may be higher. γ-Livetin, or a protein closely resembling it, also interacts with this lipovitellin–phosvitin complex and can be removed more completely by procedures that remove phosvitin. It appears, therefore, that the comparatively small phosvitin molecule may be responsible for the apparent interaction between lipovitellin and γ-livetin, resulting in the three-protein complex known classically as lipovitellin.

Acknowledgments

The authors wish to thank Mr. D. R. Muirhead for technical assistance and the Poultry Division, Canada Department of Agriculture, for supplying fresh eggs as required.

References

1. WARNER, R. C. The proteins. Vol. II. Pt. A. Academic Press, Inc., New York. 1954. p. 435.
2. MECHAM, D. K. and OLCOTT, H. S. J. Am. Chem. Soc. **71**, 3670 (1950).
3. VANDEGAER, J. E., REICHMANN, M. E., and COOK, W. H. Arch. Biochem. Biophys. **62**, 328 (1956).
4. JOUBERT, F. J. and COOK, W. H. Can. J. Biochem. and Physiol. **36**, 389 (1958).
5. MARTIN, W. G., VANDEGAER, J. E., and COOK, W. H. Can. J. Biochem. and Physiol. **35**, 241 (1957).
6. KING, E. J. Biochem. J. **26**, 294 (1932).
7. LONGSWORTH, L. G. J. Am. Chem. Soc. **74**, 4155 (1952).
8. ZEN, E-AN Geochim. et Cosmochim. Acta, **12**, 103 (1957).
9. LEA, C. H. and HAWKE, J. C. Biochem. J. **52**, 105 (1952).

THE SECRETION OF LITHIUM IN SALIVA[1]

A. S. V. BURGEN

Abstract

Lithium was secreted in the saliva at a higher concentration than it was present in the plasma. The saliva/plasma ratio was independent of the plasma concentration, but inversely related to the rate of saliva secretion. No significant concentration of lithium in the gland tissue occurred.

Introduction

In some respects the physiological behavior of lithium mimics that of sodium. For instance it can more or less substitute for sodium in nerve conduction (1, 2, 3) and it is transported in competition with sodium across frog skin (4). In other respects lithium is unique and resembles neither sodium nor potassium. As far as most body cells are concerned lithium is neither accumulated (like potassium) in the cells nor expelled (like sodium) from them, and indeed to a first approximation intracellular concentrations are very similar to the extracellular concentrations (5). The presence of lithium in saliva after intraperitoneal administration was noted by Good (6) and the present note is concerned with the quantitative nature of the lithium excretion in saliva.

Experimental

Experiments were done on mongrel dogs anaesthetized with chloralose–urethane and with the submaxillary and parotid ducts and secretomotor nerves prepared as described previously (7).

Lithium was administered by the slow infusion of a 2–10% solution of LiCl at a rate slow enough (0.1–1 ml./min.) to prevent excessive elevation of the plasma potassium (8).

Constant levels of 4–20 meq./liter Li were produced in this way without obvious ill effects.

After digestion with concentrated nitric acid the lithium concentration in plasma, saliva, and samples of salivary gland tissue was measured by flame photometry at 670.7 mμ in the Beckman DU Spectrophotometer with flame attachment. Sodium and potassium determinations were also made.

Results

Neither in the parotid nor in the submaxillary secretion was there any substantial dependence of the concentration of Li^+ in the saliva on previous secretory activity of the gland. Also, in neither gland was there an appreciable Li transient at the beginning of secretion. There was, however, in the secretion of both glands a reciprocal relationship between concentration of lithium and rate of secretion of the type illustrated in Fig. 1. At low flows the saliva Li^+ level was two to three times the plasma level and at the highest flows it

[1]Manuscript received November 28, 1957.
Contribution from the Department of Physiology, McGill University, Montreal.

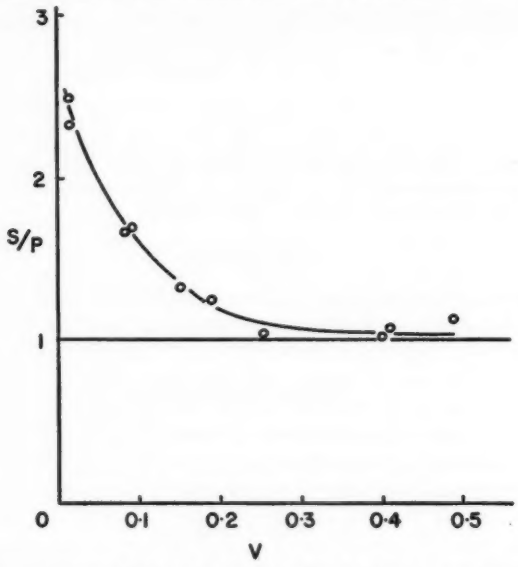


FIG. 1. Concentration of lithium in parotid saliva of the dog as a function of the rate of saliva secretion. Plasma lithium 10.1 meq./liter.
Ordinate: saliva/plasma lithium concentration ratio.
Abscissa: rate of saliva secretion ml./g. min.

approximated to the plasma level. In one instance at high rates of secretion the saliva concentration fell to as low as 70% of the plasma level. In both parotid and submaxillary saliva the ratio of saliva to plasma Li^+ concentrations appeared to be independent of the absolute plasma Li^+ level over the range tested (4.2–20.0 meq./liter).

In five experiments gland analysis was done after a constant plasma lithium concentration had been maintained for at least four hours. The ratio Li (gland water)/Li (plasma water) was 1.14 ± 0.14 (mean $\pm$ S.E.) and did not differ significantly between stimulated and non-stimulated glands.

Discussion

The pattern of secretion of lithium in the saliva differs from that of any other substance so far reported. It resembles potassium and iodide (7, 9) in usually being secreted at levels higher than those in the plasma but differs in having a much higher dependence on the rate of saliva secretion than either of these substances, in not having an appreciable time transient, and in the failure of the gland to accumulate the substance appreciably above the plasma level. The behavior of lithium is totally unlike that of sodium whose concentration in this secretion is well below that in the plasma and shows a positive dependence on the rate of saliva secretion (10, 11). At the present time no satisfactory mechanism can be postulated to explain the secretion of lithium by the salivary glands.

Acknowledgment

The work was supported by a grant from the National Research Council of Canada.

References

1. HODGKIN, A. L. J. Physiol. **106**, 319 (1947).
2. HUXLEY, A. F. and STÄMPFLI, R. J. Physiol. **112**, 496 (1951).
3. CRESCITELLI, F. Am. J. Physiol. **169**, 1 (1952).
4. ZERAHN, K. Acta Physiol. Scand. **33**, 347 (1955).
5. SCHOU, M. Pharmacol. Revs. **9**, 17 (1957).
6. GOOD, C. A. Am. J. Med. Sci. **125**, 273 (1903).
7. BURGEN, A. S. V. J. Physiol. **132**, 20 (1956).
8. MCKUSICK, V. A. J. Clin. Invest. **33**, 598 (1954).
9. BURGEN, A. S. V. and SEEMAN, P. Can. J. Biochem. and Physiol. **35**, 481 (1957).
10. GREGERSEN, M. I. and INGALLS, E. M. Am. J. Physiol. **98**, 441 (1931).
11. THAYSEN, J. H., THORN, N. A., and SCHWARTZ, I. L. Am. J. Physiol. **178**, 155 (1954).

LIBERATION OF HEXOSAMINE, HEXURONIC ACID, AND HYDROXYPROLINE FROM TISSUES BY RESIN HYDROLYSIS[1]

P. A. ANASTASSIADIS AND R. H. COMMON

Abstract

Excess sulphonated polystyrene resin (Dowex-50, 200–400 mesh) suspended in 0.05 *N* hydrochloric acid has been found suitable as a catalyst for the hydrolysis of tissue samples preparatory to the determination of hexosamine, hexuronic acid, and hydroxyproline. This technique protected hexuronic acid from destruction during hydrolysis sufficiently well to permit of its determination. The resin used in hydrolysis has been used also for separation of sugars and hexuronic acid from hexosamine and hydroxyproline by differential elution.

Introduction

Sulphonated polystyrene resins have been used as catalysts for hydrolysis of proteins (1) and carbohydrates (2, 3). Previous work in this laboratory has indicated that hexosamine may be liberated from tissue samples by hydrolysis with a suspension of sulphonated polystyrene resin (Dowex-50, 200–400 mesh) in 0.05 *N* hydrochloric acid. The hexosamine was separated from other sugars by differential elution from the resin after hydrolysis, and then determined in the appropriate eluate. The range of hydrolysis times for optimal recovery of hexosamine was found to be wide for the tissue examined, although it varied from tissue to tissue. The present paper deals with an investigation of the possibilities of using this hydrolytic technique for the determination of hexosamine, hexuronic acid, and hydroxyproline in the same hydrolyzate. These three substances are fundamental constituents of the intercellular substances (mucopolysaccharides and collagen) and their determination in the same hydrolyzate would be advantageous in studies on the glycoproteins of tissues.

In the present paper the problem of separation of hexosamine and hydroxyproline from hexuronic acid is examined in Section A. Section B presents the results of studies on the effects of time of heating and of concentration of acid on the course of resin-catalyzed hydrolysis. Section C is concerned with some major modifications of the technique of resin-catalyzed hydrolysis. Section D describes studies on the yields of hexuronic acid from resin-catalyzed hydrolysis of tissue components. Finally, Section E describes a proposed procedure for determination of hexosamine, hydroxyproline, and hexuronic acid on one and the same hydrolyzate.

Experimental

A. Separation of Hexosamine and Hydroxyproline from Hexuronic Acid and Sugars

In the method as described elsewhere (4), sugars and uronic acids were eluted from the resin after hydrolysis by draining the resin and washing it

[1]Manuscript received November 7, 1957.
Contribution from the Department of Chemistry of the Faculty of Agriculture of McGill University, Macdonald College, Que.
Macdonald College Journal Series No. 416.

with water. Losses of hexosamine and hydroxyproline in the eluate were reduced when the combined drainage and washings ("water eluate") were passed through the resin column again. Hexosamine and hydroxyproline were then eluted from the resin with 2*N* HCl. It was found, nevertheless, that there were still slight losses of hexosamine and hydroxyproline. It seems that small quantities of these substances absorbed in the lower layer of the column were eluted when the water eluate was repassed through the column. In order to eliminate these losses, it was found necessary to pass the water eluate through the column after the elution of the amino compounds by acid instead of immediately after its initial collection. Details of this step are described below in Section F.

The course of elution of hexosamine and hydroxyproline from the resin treated under the conditions employed in the analysis was traced by a fractional collection of the eluates. Glucosamine hydrochloride (1 mg.), hydroxyproline (1 mg.), and oviduct magnum (30.5 mg.) were heated, each in a separate tube, for 24 hours with 5 ml. 1:4 suspension of Dowex-50 in 0.1 *N* HCl. The resins were eluted with dilute HCl of successively greater concentrations. The eluates were collected in 1 ml. portions in a fraction collector. The fractions were analyzed for hexosamine as described elsewhere (5, 4), except that the absorbancy was read after 3 minutes instead of after 1 hour. As it is shown in Fig. 1, for the concentrations of reagents used, the color maximum is attained at once on development. After 1 hour the rate of decline of absorbancy is less, but the 3 minute interval has the advantages of (*a*) increasing the sensitivity and (*b*) practically eliminating interference by hydroxyproline. The acetylated solution is stable for 1–2 hours when cooled in running tap water and therefore as many as 20 samples may be acetylated simultaneously. The

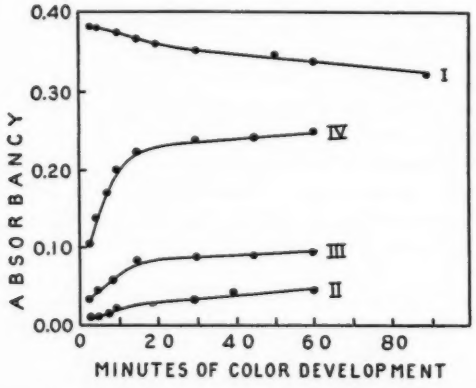


FIG. 1. Absorbancies of hexosamine and hydroxyproline at different times of color development after both substances were treated for hexosamine determination.
Curve I. Absorbancies of 4 μg./ml. final solution of hexosamine.
Curve II. Absorbancies of 50 μg./ml. final solution of hydroxyproline.
Curve III. Absorbancies of 100 μg./ml. final solution of hydroxyproline.
Curve IV. Absorbancies of 1000 μg./ml. final solution of hydroxyproline.

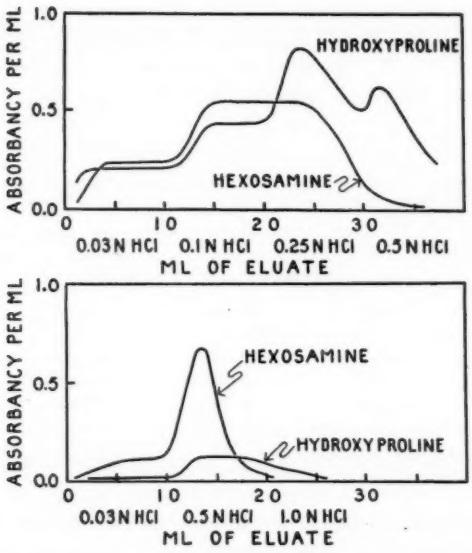


FIG. 2. Elution of hexosamine and hydroxyproline with dilute HCl from sulphonated polystyrene resin (Dowex-50). The upper pair of curves represents the elution of 1 mg. hexosamine and hydroxyproline heated separately with 1:4 suspension of resin in 0.1 *N* HCl. The lower pair of curves represents the elution of hexosamine and hydroxyproline from oviduct magnum hydrolyzed with 1:4 suspension of resin in 0.1 *N* HCl. The eluate was collected in portions each of 1 ml.

sensitivity of the method permits the determination of as little as 10 μg. hexosamine with satisfactory accuracy. Hydroxyproline was determined by the method of Neuman and Logan (6).

The results are presented in Fig. 2. It is clear that hydroxyproline was eluted more slowly than hexosamine. It should be possible, therefore, to separate the two compounds by differential elution from a column of appropriate length. Such a separation would obviate interference by hydroxyproline in the determination of hexosamine. However, the interference of hydroxyproline in the colorimetric determination of hexosamine was reduced substantially by the use of a high concentration of acetylacetone in the acetylation medium (5). The interference was further reduced to about one-third when the absorbancy was recorded shortly after the addition of the *p*-dimethylaminobenzaldehyde instead of after 1 hour (Fig. 1).

Different amounts of hydroxyproline were subjected to the color reaction for hexosamine and the absorbancies were read at 3 minutes and at 6 minutes. The absorbancy given under the same conditions by a solution 4 μg. hexosamine per milliliter final solution was also read. The data are presented in Table I. It will be noted that the error introduced by the presence of hydroxyproline will be negligible when the ratio of hydroxyproline to hexosamine is

12.5 and the time of color development is 3 minutes. A separation by differential elution is unlikely to be necessary, therefore, in the analysis of most biological materials. Furthermore, a correction can be applied readily whenever a sample is exceptionally rich in hydroxyproline.

TABLE I

INTERFERENCE OF HYDROXYPROLINE IN THE HEXOSAMINE COLOR REACTION

Hydroxyproline, μg./ml. final solution	Absorbancy		Ratio of absorbancy per μg. hydroxyproline to absorbancy per μg. hexosamine*	
	3 min.	60 min.	3 min.	60 min.
50	0.014	0.051	0.0029	0.0126
100	0.092	0.190	0.0097	0.0234
1000	0.109	0.232	0.0011	0.0029

*Absorbancy given by 4 μg. hexosamine per ml. final solution was 0.380 at 3 minutes and 0.325 at 60 minutes.

B. Effect of Time of Heating and Concentration of Dilute Acid on Resin-Catalyzed Hydrolysis of Tissue Samples

Portions (30–50 mg.) of various materials were mixed with 5 ml. of a suspension of one part Dowex-50, mesh 200–400, in four parts dilute HCl in pyrex test tubes (13 mm. × 100 mm.). The tubes were drawn out and sealed at the blow torch, and were then placed in an electric oven set to run at 107° C. The tubes were shaken by hand every 3–4 hours during heating. Tubes were removed after stated intervals, the products of hydrolysis were eluted, and hexosamine and hydroxyproline were determined in the hydrochloric acid eluates and hexuronic acid in the water eluates. Hexuronic acid was determined by the naphthoresorcinol reaction (7). Results obtained with a sample of oviduct magnum of the hen are presented in Table II. Each datum of this table is the average result of five colorimetric determinations made on each of a pair of tubes.

The hexosamine value obtained for this sample after hydrolysis with 4*N* HCl for 4 hours was 17.7 mg. per g. It is evident from Table II (*a*) that satisfactory analytical plateaux exist in the hexosamine data for hydrolysis with resin – dilute HCl in respect of both time of heating and concentration of acid; and (*b*) that the observed values are substantially the same as that obtained by hydrolysis with 4*N* HCl for 4 hours. The values for hydrolysis with resin – dilute HCl may be regarded as the more reliable in so far as this technique eliminated interference by humin formation and by sugars.

The results for hydroxyproline are also presented in Table II. It is clear that a satisfactory analytical plateau exists in respect of time of heating. In respect of concentration of acid the results indicated some destruction of hydroxyproline with 0.25 *N* HCl. The value obtained after hydrolysis with 4 *N* HCl for 4 hours was 3.63 mg. per g.

TABLE II

OBSERVED CONTENTS OF HEXOSAMINE AND OF HYDROXYPROLINE AND HEXURONIC ACID OF OVIDUCT MAGNUM OF THE HEN. HYDROLYSIS CATALYZED BY DOWEX-50 (200–400 MESH) – DILUTE HCl RATIO RESIN:ACID = 1:4

Constituent, mg. per g. dried tissue	Concn. of HCl, *N*	Time of heating			
		6 hr.	12 hr.	24 hr.	36 hr.
Hexosamine (base)	0.05	17.5	17.7	17.0	17.1
	0.10	17.7	17.5	16.6	16.6
	0.25	18.7	18.0	17.7	16.7
Hydroxyproline	0.05	–	3.74	3.74	3.97
	0.10	–	3.70	3.65	3.99
	0.25	–	2.95	2.85	3.36
Hexuronic acid	0.05	–	9.3	8.8	9.2
	0.10	–	8.8	8.8	10.0
	0.25	–	8.6	7.8	9.1

The data in Table II suggest that 0.25 *N* HCl speeded liberation of hexosamine without increasing appreciably the rate of its destruction. A working concentration of 0.05 *N* HCl was retained, however, for the following reason. A primary aim of the work was to devise a procedure for liberation of hexosamine that would permit of determination of hexuronic acid in the same hydrolyzate. Hexuronic acid is very unstable when heated with HCl. Since 0.05 *N* HCl in the resin suspension provided a satisfactory liberation of hexosamine, it was considered preferable not to use a higher concentration because of the possibility of increased destruction of hexuronic acid. A further reason for retaining 0.05 *N* HCl is the evidence of some destruction of hydroxyproline with 0.25 *N* HCl.

C. Some Modifications of the Conditions of Resin Hydrolysis

Various modifications of the conditions for the resin hydrolysis, other than the concentration of acid, were tried. The following were found to be the most satisfactory, especially as judged by high recovery of hexuronic acid. (*a*) Shaking by hand at intervals was replaced by continuous shaking in a small end-over-end shaker consisting of a rotating vertical disk to which the tubes were fastened radially by clips. The shaker was mounted inside the electric oven. The disk was connected by axles and gears (standard Meccano parts) to an electric motor mounted on top of the oven. (*b*) The temperature of the oven was reduced to 100° C. (*c*) The amount of resin was doubled, i.e., a 1:2 suspension of resin in 0.05 *N* HCl was used instead of 1:4 suspension.

Various materials were analyzed for hexosamine after they had been treated as follows:

(*a*) Hydrolysis with 4 *N* HCl for 4 hours; determination of hexosamine in total hydrolyzate.

(*b*) Hydrolysis as in (*a*); determination of hexosamine after elimination of sugars and humin by use of Dowex-50 (8).

(*c*) Hydrolysis with Dowex-50, 0.05 *N* HCl with 24 hours' heating under the modified conditions described above; determination of hexosamine in the hydrochloric acid eluate.

(*d*) Hydrolysis as in (*c*), but with 30 hours' heating.

The results are presented in Table III. Each datum in this table is the average result of at least five colorimetric determinations on each of two duplicate hydrolyzates. The results indicate that the extent of interference by sugars and humin in the tested materials was significant, as shown by the difference between colorimetry on total hydrolyzate and colorimetry after removal of humin and sugars, and that hydrolysis with Dowex-50 gave a substantially better recovery of hexosamine for most of these materials than hydrolysis with 4 *N* HCl (comparison of colorimetry after removal of humin and sugars in the 4 *N* HCl hydrolyzate with colorimetry in the HCl eluate of resin hydrolyzate).

TABLE III

HEXOSAMINE CONTENT OF VARIOUS SAMPLES DETERMINED AFTER HYDROLYSIS (*a*) WITH STRONG ACID AND (*b*) WITH SULPHONATED POLYSTYRENE PLUS 0.05 *N* HCl

	Hexosamine base, mg. per g. dried material			
	Hydrolysis with 4 *N* HCl for 4 hours		Hydrolysis with Dowex-50 plus 0.05 *N* HCl	
Material	Colorimetry on total hydrolyzate	Colorimetry after removal of humin and sugars	24 hr. heating	30 hr. heating
Secretion of prostate,* rat	22.1	17.7	18.5	19.4
Secretion of coagulating gland,* rat	54.6	36.0	51.6	47.9
Secretion of seminal vesicles,* rat	5.4	5.1	5.2	5.0
Thyroid colloid,* bovine	21.9	17.9	19.9	22.8
Casein (cow) unfractionated	2.23	1.22	1.66	–
α-Casein	2.21	1.32	1.59	–
β-Casein	2.12	1.50	1.87	–
γ-Casein	4.11	2.78	2.81	–

*Samples of materials prepared and kindly supplied by Professor C. P. Leblond. The fresh material was sectioned at 30μ on the freezing microtome. The sections were floated on saline. Cells and structural elements were sedimented on the centrifuge. The secretion remained in the supernatant, from which it was precipitated by addition of three volumes 95% ethanol. The precipitates were dried and kept in the freezer pending analysis.

D. Determination of Hexuronic Acid in Tissues after Hydrolysis by Resin – Dilute Acid

The determination of hexuronic acid in tissues without preliminary extraction of mucopolysaccharide is hampered by the ease with which hexuronic acid is decarboxylated on heating with acids during hydrolysis. Furthermore, the naphthoresorcinol reaction (7) gave obviously low results, even when

applied to purified materials, such as chrondroitin sulphate, while the carbazole reaction (9) is unreliable in the presence of proteins. It seemed possible, however, that the destruction of hexuronic acid during hydrolysis with resin – dilute HCl might be sufficiently restricted to warrant determination of hexuronic acid. Before the modifications described in Section C had been developed, portions each of 500 μg. glucuronic acid (Nutritional Biochemicals) were heated with Dowex-50 and 0.05 *N* HCl at 107° C. for various intervals. The tubes were shaken by hand every 2–3 hours. The naphthoresorcinol reaction (7) was applied to the water eluate from the resin. The results indicated that at 6 hours only 8% and at 8 hours 21% of glucuronic acid had been destroyed (Fig. 3). A hydrolysis time of 8 hours had already been found sufficient to liberate hexosamine fairly completely, at least from some materials tested. It seemed likely, therefore, that conditions might be found which would permit determination of hexosamine and hexuronic acid on the one hydrolyzate. The values for hexuronic acid obtained by the naphthoresorcinol reaction in water eluate samples and reported in Table II, although low (in view of relatively fast destruction of hexuronic acid between 6 hours and 12 hours' heating time), also suggested that hydrolysis with resin prior to determination of hexuronic acid might be feasible.

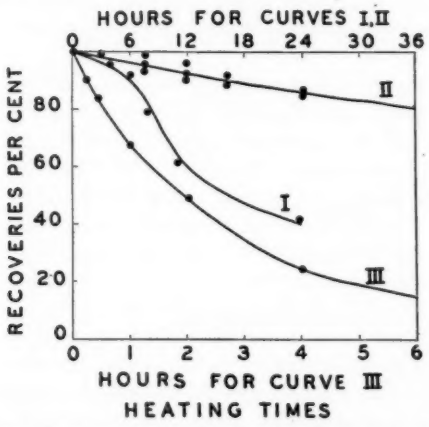


FIG. 3. Destruction of glucuronic acid as a result of heating under different hydrolytic conditions.
Curve I. Glucuronic acid was heated with sulphonated polystyrene resin suspended in 0.05 *N* HCl at 107° C. with occasional shaking.
Curve II. Glucuronic acid was heated with sulphonated polystyrene resin suspended in 0.05 *N* HCl at 100° C. with continual shaking.
Curve III. Glucuronic acid was heated with 4 *N* HCl at 100° C.

Chondroitin sulphate, known for its resistance to hydrolysis, and heparin were selected for a study of the optimal conditions for hydrolysis. Portions, 10 mg. each, of chondroitin sulphate (Nutritional Biochemicals) and of heparin (Nutritional Biochemicals) were hydrolyzed with Dowex-50 – 0.05 *N* HCl for 6 hours and 8 hours and with 4 *N* HCl for 4 hours. The hydrolyzates were

TABLE IV

HEXOSAMINE AND HEXURONIC ACID FOUND IN TWO MUCOPOLYSACCHARIDES AFTER HYDROLYSIS WITH RESIN – 0.05 *N* HCl (AT 107° C. AND WITH OCCASIONAL SHAKING) AND WITH 4 *N* HYDROCHLORIC ACID

Treatment	Chrondoitin sulphate		Heparin	
	Hexosamine (base), %	Hexuronic acid, %	Hexosamine (base), %	Hexuronic acid, %
Unhydrolyzed	–	7.9	–	2.8
Hydrolysis by resin – dil. HCl, 6 hours at 107° C.	13.5	10.0	8.5	3.5
Hydrolysis by resin – dil. HCl, 8 hours at 107° C.	15.3	10.2	12.7	3.6
Hydrolysis by 4 *N* HCl, 4 hours at 100° C.	17.4	6.6	17.7	–

analyzed for hexosamine (4) and hexuronic acid (naphthoresorcinol reaction) (7) and the results are presented in Table IV.

The values for hexuronic acid supported the opinion that satisfactory conditions of resin hydrolysis for a quantitative recovery of hexuronic acid may be found, but it was clear that under the applied conditions the glycosidic linkages of these two materials were not completely severed even after 8 hours of hydrolysis.

The modifications of the conditions of hydrolysis described above in Section C were found exceptionally promising for the recovery of hexuronic acid. Only a slight destruction of the hexuronic acid in 1 mg. samples was found after glucuronic acid had been heated with resin – 0.05 *N* HCl for the time necessary for liberation of glucuronic acid from mucopolysaccharides. This is shown by the data of Fig. 3. In this figure the course of destruction of glucuronic acid after heating with 4 *N* HCl at 100° C. for varying time intervals is also presented. A comparison of the curves for destruction of glucuronic acid when heated (*a*) with resin – 0.05 *N* HCl and (*b*) with 4 *N* hydrochloric acid is best made on the basis that the average hydrolytic time for the breakage of the glycosidic bond in mucopolysaccharides with 4 *N* hydrochloric acid is 4 hours, and that with resin – 0.05 *N* HCl under the conditions applied it is, at the most, 24 hours. It is obvious from Fig. 3 that the destruction of hexuronic acid was reduced under the new conditions of resin hydrolysis, and that it was reached at a lower order of magnitude than the destruction of glucuronic acid under conditions of strong acid.

Resin hydrolysis under the conditions described offers possibilities for the determination of hexuronic acid (simultaneously with the determination of hexosamine) of biological materials. The possibilities are illustrated by the data for chondroitin sulphate. The molecular ratio hexuronic acid:hexosamine in tissues cannot be known in advance, whereas the 1:1 ratio for chondroitin sulphate is well established. Portions each of 10 mg. chondroitin sulphate were hydrolyzed with resin for varying time intervals. The results of the analyses are presented in Table V. Most of the values are averages of

TABLE V

HEXURONIC ACID AND HEXOSAMINE CONTENTS OF COMMERCIAL CHONDROITIN SULPHATE. HYDROLYSIS BY DOWEX-50 (200–400 MESH) AND 0.05 *N* HCl AT 100° C. IN SEALED TUBES IN END-OVER-END SHAKER. ALL VALUES AS MG. PER G.

Time of heating, hr.	Hexuronic acid*			Hexuronic acid†			Hexosamine			Molecular ratio hexuronic acid: hexosamine
	Water eluate	HCl eluate	Total	Water eluate	HCl eluate	Total	Water eluate	HCl eluate	Total	
2	103	13	116	104	13	117	17	18	35	3.1
4	95	42	137	98	43	141	22	50	72	1.81
6	109	45	154	115	47	162	19	83	102	1.47
8	113	49	162	122	53	175	13	112	125	1.29
10	127	37	164	138	40	178	8	128	136	1.21
12	136	25	161	151	28	179	8	148	156	1.06
18	155	12	167	178	14	192	2	175	177	1.00
24	123	8	131	145	9	154	1	169	170	0.84
30	132	5	137	161	6	167	1	157	158	0.98

*Based on standards introduced after hydrolysis.
†Based on standards introduced before hydrolysis.

many separate hydrolyses. The highest values were observed for the 18 hours' hydrolysis time. The addition of standards before hydrolysis gave for this time the theoretical value of the molecular ratio hexuronic acid : hexosamine. In addition, the disparity between hexuronic acid values based upon standards introduced before and after the hydrolysis was small. As might be expected, the disparity was relatively greater for hydrolysis times greater than 18 hours. Very little further hexuronic acid was liberated, while its destruction continued.

The relatively high proportion of the total observed hexuronic acid in the HCl eluates after intermediate hydrolysis times (4–12 hours) might be due to an incomplete splitting of oligosaccharides at these stages. Such oligosaccharides, carrying positively charged amino groups, would tend to be held on the resin during the water elution and would be eluted only by the subsequent washing with 2 *N* HCl. Maximal values for hexosamine were observed for 18 hours' and 24 hours' heating times. The results, considered as a whole, suggest that resin hydrolysis permits a reliable determination of hexuronic acid in mucopolysaccharides when the optimal time for hydrolysis has been ascertained. The optimal time for hydrolysis for chondroitin sulphate under the given conditions was 18 hours for both hexuronic acid and hexosamine.

E. Recommended Procedure for Hydrolysis by Resin – Dilute HCl and Determination of Hexuronic Acid, Hexosamine, and Hydroxyproline in the Hydrolyzate

Weigh out 10–60 mg. of the dried (preferably freeze-dried) material containing hexuronic acid and/or hexosamine and/or hydroxyproline into a pyrex test tube 13 × 100 mm. The contents of each of the above substances in the sample should be preferably in the range 0.1–2.0 mg. Add from a pipette 5 ml. suspension of Dowex-50, 200–400 mesh, one part by weight, in 0.05 *N* HCl, two parts by volume. The suspension should be well shaken before pipetting. [The resin should be prepared by repeated washings with 2 *N* NaOH on a Büchner funnel with fritted disk M followed by several washings with 2 *N* HCl and, finally, with water.]

Seal the tubes at the blow torch and clip them to an end-over-end shaker that operates in an oven maintained at 100° C. Continue the heating for 12 to 36 hours according to the material. [If necessary, ascertain the desirable time for the particular material by carrying out hydrolysis for 12, 18, 24, and 36 hours and select the highest result.]

Remove the tubes from the oven and let them cool. Open the tubes and insert a tightly packed plug of fine pyrex glass wool. Seal the tube again, shake gently to bring the resin into suspension, and then clamp the tube firmly to a vertical position with the sealed end down. The resin will settle on the glass wool. Open the tube at the uppermost (rounded) end and break off the lowest sharp end. Collect the effluent from the tube in a 25 ml. Erlenmeyer flask. Wash the column with 11 ml. water and collect the eluate in the Erlenmeyer. The mixed primary effluent and the washings constitute

"water eluate I". [If double sealing is found inconvenient, open the tube, mix the suspension with a fine glass rod, and transfer it quantitatively with the aid of 10 ml. distilled water, to a column about 10–12 mm. diameter.]

Replace the Erlenmeyer by a 25 ml. volumetric flask (ground glass stopper). Elute amino acids with 10 ml. 2 *N* HCl. Add 5 ml. water to wash out the excess HCl and collect the washings in the volumetric. The mixture constitutes the first HCl eluate.

Replace the volumetric by a second 25 ml. volumetric. Now add to the column "water eluate I". After it has passed the column, add 11 ml. water, collecting the washings in the volumetric. Remove this volumetric, containing the "water eluate", and make to the mark with distilled water. Replace it by the volumetric that contains the first HCl acid eluate, and elute the reabsorbed hexosamine and hydroxyproline from the column with 10 ml. 2 *N* HCl. Finally, adjust the volume to the mark. Determine the hexuronic acid in the 'water eluate' by the naphthoresorcinol or carbazol reaction (7, 9). Determine hexosamine (4) and hydroxyproline (6) in the HCl eluates.

Discussion

Studies on the glycoprotein and mucopolysaccharide contents of tissue call for convenient methods of determining hexosamine and hexuronic acid in tissue samples. Hexosamine may be determined after hydrolysis with 4 *N* HCl in the traditional fashion, provided that appropriate corrections are made in the course of the colorimetric analysis or that interfering substances are eliminated through cation exchange columns. However, it has been shown previously (2), and has been confirmed in the experiments described above, that hexosamine may be determined more conveniently, and certainly more accurately, after hydrolysis with finely divided sulphonated polystyrene resin suspended in dilute (e.g. 0.05 *N*) HCl. The position as regards determinations of hexuronic acid in tissue samples has been much less satisfactory, mainly because of the rapidity with which hexuronic acids are destroyed during conventional methods of acid hydrolysis. The above experiments demonstrate that destruction of hexuronic acid during heating with resin – dilute HCl is slight as compared with that during conventional hydrolytic procedures. However, it is necessary to determine the optimal conditions for recovery of maximal amounts of hexuronic acid from hydrolyzates of any particular tissue. Imperfect separation during the elution of hexosamine and hexuronic acids may be due to the presence of oligosaccharides and may be used as a guide for judging the completeness of the hydrolysis. It is suggested that the proposed method offers a useful approach to the determination of hexosamine and hexuronic acid in tissues without prior separation of the glycoprotein or mucopolysaccharidic constituents.

Acknowledgments

The authors wish to thank Professor C. P. Leblond, McGill University, for supplying samples of certain preparations of tissue and secretions and

Mr. G. O. Henneberry, Macdonald College, for presenting samples of α-, β, and γ-casein prepared by himself. Part of the expenses was defrayed by a Grant-in-aid of Research by the National Research Council.

References

1. PAULSON, J. C. and DEATHERAGE, F. E. J. Biol. Chem. **209**, 909 (1953).
2. CLEGG, R. E., EIDINGER, D., and LEBLOND, C. P. Science, **118**, 614 (1953).
3. WADMAN, W. C. J. Chem. Soc. 3051 (1952).
4. ANASTASSIADIS, P. A. and COMMON, R. H. J. Sci. Food Agr. **4**, 229 (1955).
5. ANASTASSIADIS, P. A. and COMMON, R. H. Can. J. Chem. **31**, 1093 (1953).
6. NEUMAN, R. E. and LOGAN, M. A. J. Biol. Chem. **184**, 299 (1950).
7. HANSON, S. W. F., MILLS, G. T., and WILLIAMS, R. T. Biochem. J. (London), **38**, 274 (1944).
8. BOAS, N. F. J. Biol. Chem. **204**, 3 (1953).
9. DISCHE, Z. J. Biol. Chem. **167**, 189 (1947).

CHANGES IN SALT AND WATER DISTRIBUTION, BLOOD PRESSURE, AND ADRENAL ACTIVITY FOLLOWING NEUROHYPOPHYSEAL DENERVATION IN THE RAT[1]

SYDNEY M. FRIEDMAN, WILLIAM A. WEBBER,[2] HARALD F. SCHERRER, AND CONSTANCE L. FRIEDMAN

Abstract

Diabetes insipidus was induced by stereotaxic placement of a small lesion in the supraopticohypophyseal tract at the median eminence of the hypothalamus. The presence of diabetes insipidus was indicated by a polyuric response to an imposed salt load which, even with rigid selection criteria, occurred in a high percentage of lesion-bearing animals. In some animals the polyuric response was observed as early as the second postoperative day. No sign of remission was observed within the 4 week period of observation. Extracellular fluid volume and calculated extracellular sodium were increased in the diabetic rats. Basal blood pressure was reduced while sensitivity to norepinephrine and to pitressin was enhanced. The lesion was followed also by a reduction in gonadotrophic activity as indicated by a marked reduction in testis size.

Recently we demonstrated in both the rat (3) and the dog (5) that the acute administration of pitressin produces well-defined extrarenal effects on the distribution of sodium and water between cells and extracellular space. These findings suggest that the level of neurohypophyseal secretion may ordinarily be concerned in salt and water homeostasis at an extrarenal level. Our conclusion that neurohypophyseal secretion is concerned in the extrarenal regulation of salt and water distribution is supported indirectly by the fact that the expansion of extracellular space, a prominent feature of age (10), is, in the rat, associated with diminished function of the neurohypophysis (2,4) and directly by the fact that interruption of neurohypophyseal function produces an increase in extracellular fluid volume (6). In both cases the expansion of the extracellular space cannot be ascribed to the kidney since it can be demonstrated in the bilaterally nephrectomized animal. Since the extracellular concentration of sodium remains unchanged, the total extracellular sodium must also be increased.

The present report is concerned with a further description of the characteristics of the diabetes insipidus produced by electrolytic interruption of the supraopticohypophyseal tract. The study was carried out with the aim of providing a basic framework for further studies of the role of the neurohypophysis in blood pressure and electrolyte regulation.

Methods

Adult male albino rats of an inbred Wistar strain were used throughout. Diabetes insipidus was produced by interrupting the supraopticohypophyseal tract with a small lesion (approximately 0.5 mm. long) in the median eminence.

[1]Manuscript received October 28, 1957.
Contribution from the Department of Anatomy, The University of British Columbia, Vancouver, B.C. This work was carried out with the aid of grants from the British Columbia Heart Foundation and the National Research Council of Canada.
[2]Lederle Medical Student Research Fellow.

For this procedure, the animals were anaesthetized with nembutal–urethane and placed in a stereotaxic instrument. The skull was trephined in the midline just behind the bregma. The superior sagittal sinus was pulled laterally and a 36 gauge nichrome wire, insulated except for the tip, was lowered in the midline to the base of the brain approximately midway between the optic chiasma and mammillary bodies. Direct current, 3 ma., was applied for 12–15 seconds with an indifferent electrode inserted in the rectum.

Body weight was determined before the lesion was placed and every 2 days thereafter. The water intake for the three animals in each cage was measured daily. The presence and degree of diabetes insipidus was assessed at frequent intervals by measuring the urinary output following the administration of a salt load, 100 mg. of NaCl in 3 ml. of water, by stomach tube. Urine was collected 0.5, 1.5, 2.5, 3.5, and 4.5 hours after loading.

Tail vein blood was used for the measurement of the haematocrit and of blood glucose. Extracellular fluid volume was estimated as the inulin space determined 2 hours after the intravenous injection of a measured amount of inulin in the bilaterally nephrectomized rat as previously described (6). The concentration of sodium and potassium in arterial plasma was measured by flame photometry. Blood pressure, under light ether anesthesia, was measured directly from the femoral artery using a Sanborn electromanometer.

Experiments on two separate groups of rats, involving observation of animals for 28 days after operation, were carried out.

Experimental

Body Weight

Both test groups showed a mild weight loss during the first 12 days. By the end of the experiment, the weight loss was partly restored in the first experiment and fully restored in the second.

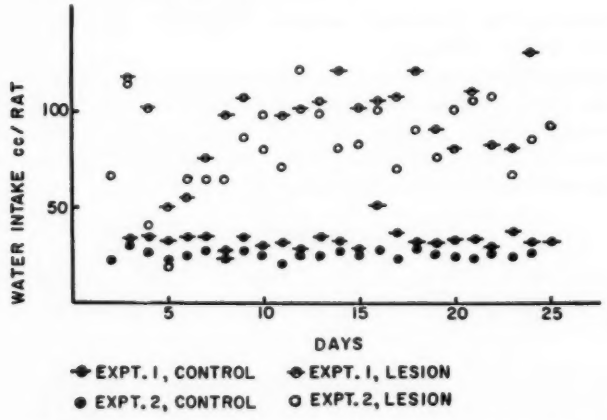


FIG. 1. Average water intake of control rats and of rats with a lesion in the supraopticohypophyseal tract in two separate experiments.

TABLE I

THE EXCRETION OF WATER FOLLOWING THE ADMINISTRATION OF 100 MG. OF NaCl IN 3 ML. OF WATER BY STOMACH TUBE IN CONTROL RATS AND IN RATS WITH A LESION IN THE SUPRAOPTICOHYPOPHYSEAL TRACT (LESION-MIXED). FROM THE LATTER GROUP, ALL RATS WITH A 4½ HOUR EXCRETION GREATER THAN TWICE THE MEAN OF THE CONTROLS WERE SELECTED AS THE DIABETES INSIPIDUS GROUP

Days postoperative	Group	No. animals	Urine excretion rate, μl./100 g./min.					Urine volume, 4.5 hr. total, ml.
			0–30 min.	30–90 min.	90–150 min.	150–210 min.	210–270 min.	
2 (Expt. 1)	Control	8	7.1±2.2	7.1±1.7	4.5±0.8	4.5±0.8	2.3±0.6	4.67±0.79
	Lesion-mixed	10	8.1±2.0	8.5±1.7	9.8±2.7	6.4±1.3	6.1±1.3*	6.25±1.17
	Lesion-diab. insip.	5	12.2±3.0	12.8±0.3*	14.5±1.3*	9.8±1.7*	9.5±1.3*	9.41±0.93*
5 (Expt. 2)	Control	8	6.2±1.4	5.9±1.0	5.2±1.0	4.9±2.1	5.2±1.0	4.21±0.68
	Lesion-mixed	9	13.5±4.6	10.0±3.1	7.0±1.2	6.2±1.2	4.6±1.2	5.31±1.84
	Lesion-diab. insip.	4	25.2±5.0*	17.4±2.7*	8.5±1.5	8.5±0.8	5.0±1.5	8.12±1.13*
8 (Expt. 1)	Control	8	3.9±0.5	5.0±0.8	3.4±0.3	2.2±0.5	2.0±0.8	3.20±0.36
	Lesion-mixed	12	12.6±1.1*	16.0±3.8*	11.4±3.0*	8.0±2.7*	7.6±1.9*	8.17±1.80*
	Lesion-diab. insip.	7	12.6±2.3*	22.5±4.6*	17.5±3.8*	12.2±3.4*	11.0±2.3*	11.01±2.14*
11 (Expt. 2)	Control	7	9.5±3.4	6.5±1.0	3.4±0.7	2.7±0.7	1.7±0.7	3.40±0.51
	Lesion-mixed	7	24.6±4.7*	17.6±3.5*	9.4±2.3*	7.4±1.9*	7.4±1.9*	8.41±1.12*
	Lesion-diab. insip.	6	26.5±4.3*	19.1±1.9*	10.1±2.3*	7.8±1.9*	8.2±1.9*	9.08±1.07*
16 (Expt. 1)	Control	8	3.0±0.3	3.8±0.5	2.4±0.5	1.1±0.3	1.6±0.3	2.39±0.28
	Lesion-mixed	9	32.0±4.0*	16.2±2.2*	13.6±3.7*	3.3±1.5	4.0±1.1	8.73±1.46*
	Lesion-diab. insip.	6	37.2±4.4*	19.8±1.8*	19.1±4.0*	4.8±1.8	5.5±1.1*	11.12±1.32*
19 (Expt. 2)	Control	8	9.2±1.6	4.6±1.0	2.9±0.6	1.6±0.3	2.0±0.6	2.92±0.47
	Lesion-mixed	6	28.1±8.2*	16.0±3.6*	11.4±2.5*	8.5±2.8*	4.3±1.1	9.28±1.95*
	Lesion-diab. insip.	5	32.0±8.5*	17.4±3.6*	12.4±2.8*	9.6±3.2*	6.0±1.1*	10.46±1.90*
25 (Expt. 1)	Control	7	16.6±0.8	11.1±0.8	3.5±0.5	1.1±0.3	1.9±0.5	5.85±0.27
	Lesion-mixed	8	32.4±6.2*	23.7±3.1*	10.0±2.1*	6.6±1.4*	4.9±1.0*	10.67±1.34*
	Lesion-diab. insip.	5	40.0±7.3*	28.2±3.5*	11.8±3.5*	8.0±1.7*	6.6±1.0*	12.91±1.27*

NOTE: ± = Standard error.
* = $p < 0.02$.

TABLE II

THE EXCRETION OF SODIUM FOLLOWING THE ADMINISTRATION OF 100 MG. OF NaCl IN 3 ML. OF WATER BY STOMACH TUBE IN CONTROL RATS AND IN RATS WITH A LESION IN THE SUPRAOPTICOHYPOPHYSEAL TRACT SELECTED AS DIABETIC (4½ HOUR WATER EXCRETION GREATER THAN TWICE THE MEAN OF THE CONTROLS). SIGNIFICANCE IS INDICATED ONLY FOR INDIVIDUAL PERIODS

Days postoperative	Group	No. animals	Sodium excretion rate, μeq./100 g./min.				
			0–30 min.	30–90 min.	90–150 min.	150–210 min.	210–270 min.
2 (Expt. 1)	Control	8	1.1±0.2	1.9±0.4	1.1±0.3	0.7±0.1	0.6±0.2
	Diabetes insipidus	5	0.3±0.2*	0.9±0.4	1.6±0.4	0.9±0.1	0.8±0.1
5 (Expt. 2)	Control	8	1.9±0.4	1.5±0.3	1.3±0.2	0.8±0.2	1.1±0.2
	Diabetes insipidus	4	4.7±1.2	3.9±0.3*	1.8±0.3	1.6±0.4	1.0±0.2
8 (Expt. 1)	Control	8	0.9±0.2	1.4±0.2	0.9±0.1	0.8±0.1	0.6±0.2
	Diabetes insipidus	6	0.9±0.3	2.4±0.5	2.0±0.5	1.3±0.2	0.7±0.1
11 (Expt. 2)	Control	7	1.9±0.6	2.1±0.4	0.9±0.2	1.1±0.4	0.7±0.2
	Diabetes insipidus	7	3.3±0.9	3.2±0.4	1.6±0.2	1.2±0.3	1.6±0.4
16 (Expt. 1)	Control	8	1.1±0.3	1.4±0.2	0.9±0.1	0.7±0.2	0.6±0.1
	Diabetes insipidus	5	5.0±1.1*	4.0±0.7*	1.9±0.4*	0.7±0.1	0.8±0.2
19 (Expt. 2)	Control	8	2.2±0.4	1.7±0.3	1.1±0.2	0.5±0.1	0.7±0.2
	Diabetes insipidus	6	2.8±0.8	3.2±0.6	1.8±0.2*	1.5±0.4*	0.9±0.2
25 (Expt. 1)	Control	8	3.1±0.4	1.8±0.2	1.0±0.1	0.5±0.1	0.5±0.1
	Diabetes insipidus	6	3.5±1.1	3.4±0.5*	1.2±0.2	1.0±0.1*	0.9±0.1*

NOTE: ± = Standard error.
* = $p < 0.02$.

Fluid Intake

The crude pattern of water intake is shown in Fig. 1. Since this is based on group intake, no analytic calculation can be made. Two points, however, are evident: (1) water intake was markedly elevated in both test groups and (2) there was a strong suggestion of an interphase with reduced intake between the 4th and 7th postoperative days.

Water Excretion after Salt Load

In this test for the presence of diabetes insipidus, the lesion-bearing animals were easily subdivided into those without and those with a diabetic response; the urine volume was either well within the normal range for the simultaneously treated controls or far beyond it. With time, some previously nondiabetic animals became diabetic (Table I); the reverse did not occur. The pattern of response differentiates the diabetic animals as early as 2 days postoperatively and still more sharply 5 and 8 days after operation.

Sodium Excretion after Salt Load

Table II compares the sodium excretion of the control and diabetic groups. When the response to a salt load was measured 2 days after operation there was a sharply reduced sodium output despite the presence of a well-defined polyuric response. Beginning 5 days after operation, however, there was a consistent increase in sodium excretion which was approximately proportional to the increase in water excretion.

TABLE III

BLOOD PRESSURE SENSITIVITY AND ELECTROLYTE DISTRIBUTION IN CONTROL RATS AND IN RATS WITH A LESION IN THE SUPRAOPTICOHYPOPHYSEAL TRACT SELECTED AS DIABETIC (4½ HOUR WATER EXCRETION GREATER THAN TWICE THE MEAN OF THE CONTROLS)

	Experiment 1		Experiment 2	
	Control	Diabetes insipidus	Control	Diabetes insipidus
Blood pressure				
Systolic	114±6	80±4*	126±7	91±8*
Diastolic	76±5	52±5*	95±6	62±9*
Blood pressure rise after 1 μg. norepinephrine				
Systolic	+57±4	+76±4*	+51±4	+78±7*
Diastolic	+46±3	+57±3*	+44±4	+61±6*
Blood pressure rise after 20 mU. pitressin				
Systolic	+24±4	+42±8*	+21±3	+36±7
Diastolic	+20±4	+33±8	+17±3	+30±5
Blood glucose, mg.%	113±6	110±6		
Extracellular fluid volume, ml./100 g.	20.6±0.5	22.5±0.6†		
Plasma Na, meq./l.	153±1	152±3	145±4	142±4
Plasma K, meq./l.	4.1±0.2	3.3±0.1*	4.3±0.2	3.9±0.3
Haematocrit	52±1	48±2		
Adrenal wt., mg./100 g.	8.6±0.4	12.7±0.8*	10.7±0.7	13.7±0.3*
Testis wt., mg./100 g.	799±24	418±134*,‡	960±50	530±90*
No. of animals	10	6	7	5

NOTE: ± =Standard error.
* = $p < 0.02$.
† = $p < 0.05$.
‡ One rat with diabetes insipidus had a testis wt. of 1079 mg./100 g.

Blood Pressure

Blood pressure (Table III) was reduced in the presence of diabetes insipidus. At the same time, the diabetic animals showed increased sensitivity to the pressor effect of both norepinephrine and pitressin.

Blood Sugar

This was unchanged.

Sodium, Potassium, and Water

The extracellular fluid volume was elevated in the presence of diabetes insipidus, while plasma sodium concentration remained unchanged—a combination signifying an actual increase in total extracellular sodium (Table III). The observed fall in plasma potassium concentration is at least in part referable to the increase in extracellular fluid, as is the tendency for the haematocrit to fall.

Organ Weights

Heart, kidney, thyroid, adrenal, and testis weights were determined. The adrenal was found to be enlarged and the testis reduced.

Discussion

Interruption of the supraopticohypophyseal tract is an effective means of producing diabetes insipidus in the rat. The small lesion placed in the median eminence as described produces the syndrome in a high percentage of cases (75–90% in our experience). In most animals, the condition is manifest within 7 to 10 days of operation; in a few it becomes evident during the third week. Presumably, such a small lesion may occasionally leave untouched a few functioning elements which may or may not survive the ensuing ascending degeneration from the site of section. There is a suggestion of an interphase between the 4th and 7th postoperative days.

In agreement with Bogdanove's mapping of the anterior hypothalamus (1) interruption of the tract as described also involves a loss of gonadotrophin production. There is, however, no reason to assume that the diabetic response is related to this unavoidable overlap destruction. From the standpoint of general suitability for the investigation of diabetes insipidus this method is less complicated by anterior pituitary dysfunction than classical stalk section and, in contrast with neurohypophysectomy, is more regularly permanent (8).

The salt loading test is, as described by others (7), a convenient way of differentiating those animals with even minimal diabetes insipidus. Our findings with timed urine collections extending over a 4½ hour period indicate that a single 2 hour collection period would provide adequate differentiation. The excretion of sodium is regularly increased together with water as reported by Jones (9). At the 2 day interval, however, sodium excretion was reduced despite the polyuric response to the load.

The increase in extracellular fluid and consequently in calculated total extracellular sodium which we have previously reported in the 10 day postoperative diabetic rat still persists here 4 weeks after operation. The shift

in fluid and salt distribution in the previous study was demonstrated in animals deprived of water for 24 hours and of their kidneys for 20 hours (6). Presumably in the present experiments the shifts are still independent of the kidney.

The absence of neurohypophyseal function leads to a more prominently displayed adrenal cortical activity as demonstrated by the fact that the increase in fluid volume in diabetes insipidus can largely be reversed by adrenalectomy even in the absence of the kidneys (6). It is also demonstrated by the adrenal enlargement observed here and described in detail by Jones (9).

The reduction in blood pressure following neurohypophyseal denervation is consistent and reproducible. It accords in general with our theory that blood pressure regulation depends on the sodium transfer systems (3, 5) and obviously these are deranged here, but a specific explanation cannot yet be attempted.

References

1. BOGDANOVE, E. M. Endocrinology, **60**, 689 (1957).
2. FRIEDMAN, S. M. and FRIEDMAN, C. L. Gerontologia, **1**, 107 (1957).
3. FRIEDMAN, S. M., FRIEDMAN, C. L., and NAKASHIMA, M. Circulation Research, **5**, 261 (1957).
4. FRIEDMAN, S. M., HINKE, J. A. M., and FRIEDMAN, C. L. J. Gerontol. **11**, 286 (1956).
5. FRIEDMAN, S. M., BUTT, R. M., and FRIEDMAN, C. L. Am. J. Physiol. **190**, 507 (1957).
6. FRIEDMAN, S. M., SCHERRER, H. F., NAKASHIMA, M., and FRIEDMAN, C. L. Am. J. Physiol. In press.
7. HARE, R. S., HARE, K., and PHILIPS, D. M. Am. J. Physiol. **140**, 334 (1943).
8. HARRIS, G. W. Neural control of the pituitary gland. Edward Arnold & Co., London. 1955.
9. JONES, I. C. *In* The neurohypophysis. *Edited by* H. Heller. Butterworth Scientific Publications, London. 1957. p. 253 et seq.
10. LOWRY, O. H. and HASTINGS, A. B. *In* Cowdry's Problems of ageing. 3rd ed. Williams & Wilkins Co., Baltimore, Md. 1952. Chap. 5.

THE EFFECT OF DIETARY OILS AND FATTY ACIDS ON CHOLESTEROL METABOLISM IN THE RAT[1]

J. D. WOOD[2] AND B. B. MIGICOVSKY

Abstract

Rats were fed diets containing 20% oils and 9% fatty acids and the effect of these compounds on cholesterol metabolism was studied. Unsaturated oils and fatty acids increased total cholesterol in the liver and stimulated the incorporation of C^{14}-acetate into cholesterol both *in vivo* and in liver homogenates. Saturated material such as coconut oil and lauric acid had the opposite effect with respect to amount of liver cholesterol and to *in vivo* incorporation. The saturated material had no significant effect on synthesis in homogenates. The effect of oils in the diet was rapid, the stimulating effect of rapeseed oils being observed after the rats had been placed on the diet for as short a period as 3 days.

Introduction

The type of diet consumed by animals has a direct bearing on the level of cholesterol in tissues. Okey and Stone (1) showed that rats fed a diet containing 15% lard tended to store more liver cholesterol than rats fed a similar diet in which cottonseed oil replaced lard. Carroll (2) stated that rapeseed oil at a level of 25% in the diet increased the amount of cholesterol in adrenals and liver. The same worker (3) showed that a similar effect was obtained by feeding erucic acid, one of the component fatty acids of rapeseed oil.

Previous studies in our laboratory (4) showed that when fatty acids were added directly to rat liver homogenates they brought about a decrease in the C^{14}-acetate incorporation into cholesterol.

It therefore appeared desirable to find out if feeding various oils and fatty acids had any influence on the synthesis of cholesterol from acetate by rat tissues. Synthesis both *in vivo* and *in vitro* was studied and the results of these investigations are presented in this paper.

Materials and Methods

Animals

The rats were males weighing 100 to 130 g., the weight range being kept as small as possible within any one experiment.

Diets

The control diet was crushed Purina Fox Chow cubes which contained 22% protein, 3.6% fat, and 4.2% fiber. The amount of digitonide-precipitable sterols was approximately 0.06%. Experimental diets were prepared by mixing 20% of the individual oils or 9% of the fatty acids with the control diet. The animals were kept on these diets for 7 days unless otherwise stated.

[1]Manuscript received November 29, 1957.
Contribution No. 384, Chemistry Division, Science Service, Canada Department of Agriculture, Ottawa.

[2]Present address: Technological Station, Fisheries Research Board of Canada, 898 Richards Street, Vancouver 2, B.C.

Cholesterol Synthesis in vitro

Homogenates were prepared from livers by the method of Bucher (5) with the following adaptations. The liver was perfused with ice-cold 0.25 *M* sucrose prior to excision, and nicotinamide and $MgCl_2$ were omitted from the buffer solution and added directly to the incubation flasks.

Incubations were carried out in a Dubnoff water bath under oxygen at 37° C. for a period of 2.5 hours. The incubation mixture consisted of 2.0 ml. liver homogenate, 1.3 mg. ATP, 3.8 mg. DPN, 8 μM. C^{14}-acetate, 2 μM. $MgCl_2$, 10 mg. nicotinamide, and water to give a final volume of 2.8 ml.

The C^{14}-incorporation into cholesterol was measured by the method of Rabinovitz and Greenberg (6).

Cholesterol Synthesis in vivo

Rats were dosed orally with 1.0 ml. of an aqueous solution containing 10 μM. C^{14}-acetate. The animals were killed 2 hours after dosing and the liver was excised, weighed, and homogenized in about 15 ml. distilled water. The total volume of the homogenate was made up to 25 ml. with water.

A 10-ml. aliquot of the homogenate was digested with 10 ml. ethanol and 1.5 g. potassium hydroxide in a boiling water bath overnight. Cholesterol was extracted from the solution and radioactivity measured by the same method as used in the *in vitro* studies. The amount of C^{14} incorporated into 1 mg. of cholesterol was calculated.

The total cholesterol estimations were carried out on 1.0 ml. samples of the homogenate using the Liebermann-Burchard reagent by the method described by Schoenheimer and Sperry (7) and Sperry and Webb (8) except that six drops of KOH were used for the digestion with 6 ml. of the acetone–ethanol extract and no further addition of acetone–ethanol was made.

The total amount of C^{14} incorporated into liver cholesterol was calculated from the above data.

The selection of a 2-hour interval between acetate dosage and sampling of the liver is based on an experiment in which rats were dosed with C^{14}-acetate and killed for liver sampling at various time intervals thereafter. The results of this experiment, shown in Fig. 1, indicate that incorporation of acetate into liver cholesterol is quite rapid and reaches a plateau within 60 minutes and the plateau persists beyond the 2-hour interval.

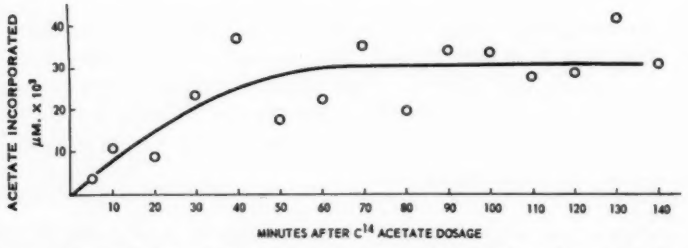


FIG. 1. Incorporation of C^{14}-acetate into liver cholesterol at intervals after C^{14}-acetate administration.

Identification of Liver Sterols

A 100-ml. portion of rat liver homogenate was saponified overnight with 150 ml. ethanol and 15 g. KOH. Sterols were extracted twice with ether, and the extract was washed with distilled water twice, with dilute HCl, and finally with distilled water. The solution was dried over Na_2SO_4 and evaporated to remove ether. The dry unsaponifiable matter was dissolved in petroleum ether and the sterol fraction was separated and identified by the method of Coleman *et al.* (9), using column chromatography and colorimetric methods.

Results

Studies were made on the incorporation of C^{14}-acetate into cholesterol in liver homogenates of rats which had been kept on different diets for a period of 7 days. The results are shown in Table I. The C^{14}-incorporations tabulated are the mean values for four rats. An analysis of variance of the data was calculated.

TABLE I

EFFECT OF DIFFERENT OILS AND FATTY ACIDS IN THE DIET ON THE C^{14}-ACETATE INCORPORATED INTO CHOLESTEROL BY LIVER HOMOGENATES

Diet	C^{14}-acetate incorporated (μM. $\times 10^4$)	
	Experiment A	Experiment B
Control	12	53
Rapeseed oil**	401	690
Corn oil**	417	400
Coconut oil	45	66
Control	70	11
Erucic acid**	606	202
Oleic acid**	541	135
Lauric acid	274	18

**Analysis of variance indicates difference from control is significant at $P = .01$.

The addition of rapeseed oil or corn oil to the diet had a stimulating effect on C^{14}-incorporation into cholesterol, and this was significant at the 1% level in spite of the wide variation between animals in the same group. The main component fatty acids of rapeseed oil and corn oil are erucic acid and oleic acid respectively, and these unsaturated acids are seen to have the same stimulating effect as the oils on C^{14}-incorporation. When a saturated oil or fatty acid such as coconut oil or lauric acid was introduced into the diet no significant change was observed in C^{14}-incorporation.

Groups of four rats each were placed on the control diet and the rapeseed oil-supplemented diet for varying lengths of time. Table II shows the C^{14} incorporated into cholesterol by liver homogenates from these rats. A noticeable increase in the incorporation occurred after only 3 days on the rapeseed oil diet. Similar increased incorporations were observed after 7 and 14 days

on the diets. Although the length of time on the fat diet varied, the age of the rats was the same at the time they were taken for analysis. Table II also shows C^{14}-incorporations by preparations from rats which were weaned directly onto the experimental diets and kept on these diets for 28 days. As previously, it was found that homogenates from rats fed the rapeseed oil diet incorporated C^{14}-acetate to a greater degree than did homogenates from control rats.

Table III shows C^{14}-incorporation into cholesterol after dosing with C^{14}-acetate. The results parallel those of *in vitro* studies in that rats fed unsaturated materials such as rapeseed oil, corn oil, erucic acid, and oleic acid showed an increase in the C^{14}-incorporation over the rats fed the control diet. Variation between rats of the same group was large but the effect of oils and acids

TABLE II

CHOLESTEROL SYNTHESIS IN LIVER HOMOGENATES FROM RATS FED RAPESEED OIL[1] FOR VARYING LENGTHS OF TIME[2]

Length of time on diet, days	C^{14}-acetate incorporated (μM. $\times 10^4$)	
	Control	Rapeseed oil**
3	126	697
7	47	646
14	17	585
28	14	757

[1]20% of the diet.
[2]Each value represents the mean of four rats.
**Analysis of variance indicates difference from control is significant at $P = .01$.

TABLE III

THE EFFECT OF DIETARY OILS AND FATTY ACIDS ON THE LEVEL OF LIVER CHOLESTEROL AND ON THE SYNTHESIS OF CHOLESTEROL *in vivo*[1]

Diet	C^{14} incorporated (μM. $\times 10^4$)	Liver weight, g.	Liver cholesterol, mg.
Control	140 ± 15	5.7	11.5 ± 0.4
Rapeseed oil	239 ± 16**	5.6	14.0 ± 0.7**
Corn oil	233 ± 13**	5.0	14.5 ± 0.4**
Control	324 ± 32	4.7	10.3 ± 0.4
Coconut oil	213 ± 36*	4.7	8.9 ± 0.5*
Control	176 ± 15	6.8	11.7 ± 0.3
Erucic acid	279 ± 19**	6.8	13.3 ± 0.3**
Control	226 ± 15	6.2	13.6 ± 0.3
Oleic acid	318 ± 17**	6.5	16.0 ± 0.6**
Control	251 ± 30	5.4	13.6 ± 0.6
Lauric acid	113 ± 18**	4.9	9.4 ± 0.4**

[1]Each value in the table is the mean for eight rats. The standard error of the mean is included.
*Difference from control is significant at $P = .05$.
**Difference from control is significant at $P = .01$.

was significant at the 1% level. The saturated compounds, coconut oil and lauric acid, brought about a decrease in the acetate incorporation. However coconut oil did not alter the incorporation to the same extent as did the other oils and acids.

Table III also shows liver weight and total cholesterol in the liver of rats which were fed the diets for 7 days. Unsaturated oils and fatty acids increased the level of total cholesterol in the liver, whereas saturated compounds brought about a decrease in the amount of total cholesterol. As previously, results with coconut oil were not so conclusive as those with other oils and fatty acids. It is seen from the table that the different diets, with perhaps the exception of that containing lauric acid, had little effect on the weight of the liver in the rat, and that there was no apparent connection between liver weights and total cholesterol of livers.

Sterols other than cholesterol give a color with the Liebermann-Burchard reagent, which was used to estimate total cholesterol in liver samples. Coleman *et al.* (9) showed that rat feces contain a number of these sterols. Liver sterols were therefore analyzed to ascertain whether there were other sterols present which would interfere with the cholesterol estimation. The method described earlier in this paper was used. Only one band was located on the celite – silicic acid column by spotting with the Liebermann-Burchard reagent. The color developed was typical of the cholesterol-containing band when sterols from rat feces were used as a reference. When the livers were taken from rats which had previously been dosed with C^{14}-acetate, all radioactivity on the column was contained in the band mentioned above. The material in the band was eluted and the color development with the Liebermann-Burchard reagent was studied over a period of 50 minutes and compared with

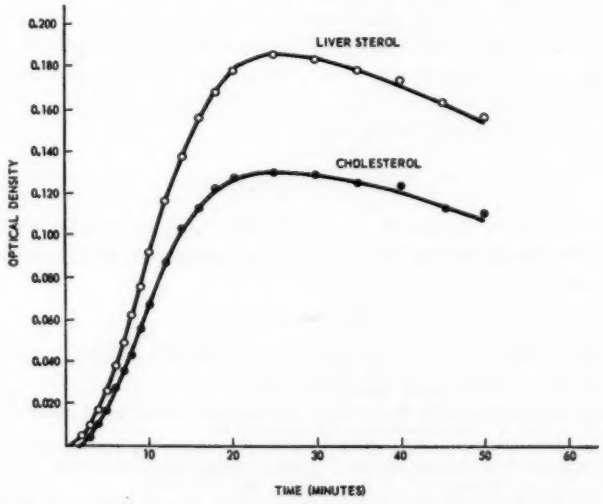


FIG. 2. Comparison of color development of liver sterol and cholesterol.

the color developed using a known sample of cholesterol. The results are shown in Fig. 2. The curves are identical in shape, indicating that cholesterol was the only sterol in the rat livers which gave a color with the reagents employed.

Discussion

It has been shown that feeding 20% rapeseed oil and corn oil in the diet has a direct bearing on the cholesterol metabolism in the rat. The amount of the total cholesterol in the liver is elevated and the amount of acetate incorporated *in vivo* into liver cholesterol is increased. The former result is in agreement with the work of Carroll (2), who showed the same increase in liver and adrenal cholesterol after feeding 25% rapeseed oil. Coconut oil, which is saturated oil, has the opposite effect.

Erucic, oleic, and lauric acids are the main component fatty acids of rapeseed oil, corn oil, and coconut oil respectively, and these acids were shown to have the same effect on cholesterol metabolism of the rat as had the corresponding oils. It would appear, therefore, that the effect of the oils is due to their fatty acid moieties.

Results of *in vitro* measurements of acetate incorporation parallel those obtained in *in vivo* studies. Unsaturated oils and acids stimulated C^{14}-acetate incorporation into cholesterol. However coconut oil and lauric acid caused no significant change in the C^{14}-acetate incorporation. These results are in contrast to those reported previously by Wood and Migicovsky (4) in which the acids were added directly to the incubation mixture. In that instance both saturated and unsaturated fatty acids were found to inhibit the C^{14}-acetate incorporation into cholesterol. It must be concluded, therefore, that the effect of adding the fatty acid directly to the homogenate is the result of a phenomenon different from that observed when the acid is fed to the animal. It appears, nevertheless, that the effect, on cholesterol synthesis, of a treatment such as dietary fat given to the live animal, can be observed by measuring acetate incorporation *in vitro*. This is based on the parallel results obtained with the *in vivo* and *in vitro* measurements.

Acknowledgments

The authors wish to thank Mr. M. L. Moore for his technical assistance, and the staff of the Small Animal Laboratory for preparing and feeding the diets to the animals.

References

1. Okey, R. and Stone, M. M. J. Am. Dietetic Assoc. **32**, 807 (1956).
2. Carroll, K. K. Endocrinology, **48**, 101 (1951).
3. Carroll, K. K. J. Biol. Chem. **200**, 287 (1953).
4. Wood, J. D. and Migicovsky, B. B. Can. J. Biochem. and Physiol. **34**, 861 (1956).
5. Bucher, N. L. R. J. Am. Chem. Soc. **75**, 498 (1953).
6. Rabinovitz, M. and Greenberg, D. M. Arch. Biochem. Biophys. **40**, 472 (1952).
7. Schoenheimer, R. and Sperry, W. M. J. Biol. Chem. **106**, 745 (1934).
8. Sperry, W. M. and Webb, M. J. Biol. Chem. **187**, 97 (1950).
9. Coleman, D. L., Wells, W. W., and Baumann, C. A. Arch. Biochem. Biophys. **60**, 412 (1956).

THE PHASE TEST INTERMEDIATE AND THE ALLOMERIZATION OF CHLOROPHYLL *a*[1]

A. S. HOLT

Abstract

The phase test intermediate of methyl chlorophyllide *a* is generated in organic solvents by alkoxyl and hydroxyl ions. The intermediate is oxidized by oxygen and such oxidants as iodine, *p*-benzoquinone, and potassium permanganate. Different products are obtained depending on the solvent, the base used to generate the intermediate, and the oxidant. Three fractions are obtained from methanol containing oxygen and traces of magnesium methoxide. These are (1) Mg-purpurin 7-trimethyl ester, (2) Mg-purpurin 7-lactone-methyl ether-dimethyl ester, and (3) Mg-10-oxy-methyl pheophorbide *a*. The first and third fractions each make up 10–15% of the total oxidized pigment. Mg-"unstable" chlorin diester is obtained by oxidation with potassium permanganate of the phase test intermediate generated in pyridine by aqueous potassium hydroxide. Mg-10-methoxy-methyl pheophorbide *a* is obtained by oxidation with iodine of the phase test intermediate generated in methanol by magnesium methoxide (*ca.* 7%, w/v). It is concluded that allomerization of chlorophyll in alcoholic solution is due to oxidation by oxygen of traces of phase test intermediate.

The visible and infrared absorption spectra of the allomerized derivatives are presented and discussed.

Introduction

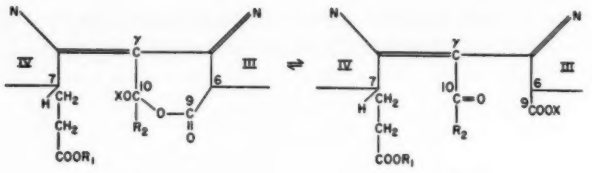
Strain has reported that the principal product of enzymatic oxidation of chlorophyll *a* in methanol extracts of barley leaves is identical with that produced by allomerization (oxidation by oxygen) in methanol (33). This and other products isolated chromatographically were characterized only by their "red" absorption maxima in different solvents. Fischer and Pfeiffer (14) have identified the magnesium-free derivative of the chief product of allomerization of chlorophyll *a* as purpurin 7-lactone-ether diester (see Fig. 1). Other derivatives have been prepared but likewise have been characterized only as magnesium-free compounds (4, 5, 13, 18).

This communication describes the preparation and the chemical and spectral properties of allomerized phyllins, some of which were encountered in extracts of killed leaves, e.g. during the preparation of methyl or ethyl chlorophyllides (24). This study supports the hypothesis (35) that the course of allomerization ordinarily involves oxidation of traces of the Molisch phase test intermediate, which is in turn generated by traces of alkali in solution. A discussion of preliminary findings of this work has appeared elsewhere (29).

Materials and Methods

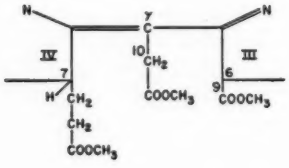
All solvents (reagent grade) except methanol were used without further treatment. For some experiments methanol was dried by refluxing over magnesium methoxide and by distilling through a silvered, vacuum-jacketed

[1] Manuscript received September 3, 1957.
Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa 2, Canada.
Issued as N.R.C. No. 4661.

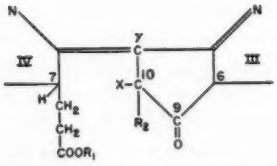


A B

COMPOUND	FIG.	X	R_1	R_2
"UNSTABLE" CHLORIN MONOESTER	A	H	H	$C(=O)OCH_3$
"UNSTABLE" CHLORIN DIESTER	"	"	CH_3, C_2H_5, etc.	"
PURPURIN 7-LACTONE-ETHER-DIESTER	"	CH_3, C_2H_5,	" "	"
PURPURIN 7-TRIESTER	B	" "	" "	"
PURPURIN 7-DIESTER	"	H	" "	"
PURPURIN-18-MONOESTER	A	ABSENT	" "	ABSENT



CHLORIN E_6-TRIMETHYL ESTER



COMPOUND	X	R_1	R_2
METHYL PHEOPHORBIDE A	H	CH_3	$C(=O)OCH_3$
10-OXY-METHYL PHEOPHORBIDE A	HO	"	"
10-METHOXY-METHYL PHEOPHORBIDE A	CH_3O	"	"

FIG. 1. Structures of chlorophyll *a* derivatives (13, 14).

column (400 mm. × 12½ mm. I.D.) packed with glass spirals (1¼ turn, 3/32 in I.D.). The magnesium concentration in the magnesium methoxide solution used for some experiments as a stock solution was 18 mg. per ml. (27).

Methyl chlorophyllide *a* was used so that the acid numbers and the methoxyl values of the oxidized derivatives could be compared to those given by Fischer (13). Because the purification of methyl chlorophyllide *a* was more difficult and time consuming than that of chlorophyll *a*, the latter pigment was used for other experiments as much as possible. The aqueous hydrochloric solutions used in the determination of acid numbers were standardized by a Twaddell hydrometer. Methoxyl determinations were made by the method of Clarke (3).

Allomerized derivatives were purified by adsorption on sucrose columns from mixtures (v/v) as 2% pyridine in petroleum ether (b.p. 30–60° C.), 10% chloroform in petroleum ether, or 20% ethyl ether in petroleum ether followed by treatment with isopropanol (0.1 to 1.5% in petroleum ether), or benzene (20–90% in petroleum ether), or sometimes benzene alone. The desired bands were separated from those of contaminants (revealed by their color or fluorescence under ultraviolet light) by elution or by carefully slicing the sugar after its removal from the glass column.

Magnesium-free derivatives were prepared by adding a small volume of 10% (w/v) aqueous HCl to an acetone solution of the phyllin followed by mmediate transfer into ethyl ether. Free acids were prepared by hydrolysis of the ester in 30% (w/v) HCl (13). After the pigment had been returned to ether, it was re-extracted into aqueous sodium carbonate solution (0.5%, w/v), and returned into fresh ether by addition of dilute hydrochloric acid.

Visible absorption spectra of ether solutions were measured with a Model DU Beckman Spectrophotometer or a Cary Recording Spectrophotometer, Model 11M. Infrared spectra were measured with a Perkin–Elmer Model 21 Double Beam Recording Spectrophotometer. The ordinates of the infrared spectra have been changed from % absorption to % transmittance in order to permit easier comparisons with spectra of chlorophyll and its derivatives published earlier (25).

Pertinent data from the visible absorption spectra of the various compounds are summarized in Table I.

Experimental Results

1. Conditions for the Phase Test

The test as described by Molisch (28) consists of layering 30% methanolic KOH (w/v) under an ether solution of pigment. At the area of contact a yellow-brown ring appears if the cyclopentanone ring has not been oxidized, or the carbomethoxy group at C_{10} has not been removed. It is thought that the yellow-brown intermediate is either an enolate ion or an ionized diradical resulting from ionization of the H atom at C_{10} (16, 35). If the mixture is shaken all pigment is extracted into the alkaline layer and in a short time

TABLE I

WAVELENGTHS OF ABSORPTION MAXIMA AND MINIMA OF CHLOROPHYLL DERIVATIVES

R = QUOTIENT OF ABSORBANCE AT BLUE MAXIMUM DIVIDED BY THE ABSORBANCE AT WAVELENGTHS INDICATED

Compound	mμ	R	mμ	R	mμ	R	mμ	R	mμ	R	mμ	R	mμ	R
Maxima														
Mg-methyl pheophorbide *a*	660	1.33	613	8.13	575	14.4	531	26.9	497	51.4			429	1.00
Mg-10-oxy-methyl pheophorbide *a*	660	1.44	614	9.03	572	16.2	530	23.5	496	46.7			429	1.00
Mg-10-methoxy-methyl pheophorbide *a*	661	1.25	613	8.55	571	15.1	531	21.5	496	36.0			429	1.00
Mg-purpurin 7-trimethyl ester	670	2.71			575	11.6	526	28.2	493	41.3			420	1.00
Mg-purpurin 7-lactone-methyl ether-dimethyl ester	652	1.88	608	11.7	565	22.8	523	43.7	492	72.2			417	1.00
Mg-"unstable" chlorin-dimethyl ester	650	1.90	607	12.9	564	25.2	523	45.3	490	71.0			417	1.00
Mg-purpurin 18-methyl ester	676	1.63	628	8.38	565	13.1	525	32.4	490	55.3			422	1.00
Mg-chlorin e_6-trimethyl ester	641	2.57	600	16.7	566	39.0	523	33.5	495	92.0			416	1.00
Methyl pheophorbide *a*	666	2.05	610	14.9	560	47.8	535	11.8	505	10.0	470	30.4	408	1.00
10-oxy-methyl pheophorbide *a*	667	2.05	609	16.4	559	43.8	532	15.3	504	9.45	467	30.4	408	1.00
10-methoxy-methyl pheophorbide *a*	667	1.96	611	16.2	561	49.6	532	14.7	503	9.41	469	29.3	408	1.00
Minima														
Mg-methyl pheophorbide *a*	633	13.1	588	20.7	548	36.4	507	52.7	473	88.0				
Mg-10-oxy-methyl pheophorbide *a*	633	13.6	587	22.4	548	40.5	507	59.3	473	93.0				
Mg-10-methoxy-methyl pheophorbide *a*	632	13.1	586	24.5	546	38.0	507	38.4	473	68.2				
Mg-purpurin 7-trimethyl ester			591	13.4	546	37.3	506	43.3	479	47.0				
Mg-purpurin 7-lactone-methyl ether-dimethyl ester	627	16.5	582	35.0	544	54.7	500	73.5	471	127				
Mg-"unstable" chlorin-dimethyl ester	625	17.9	580	40.3	543	60.6	499	74.8	469	121				
Mg-purpurin 18-methyl ester	648	13.8	600	31.2	545	58.0	504	66.5	473	80.8				
Mg-chlorin e_6-trimethyl ester	615	19.3	572	43.0	546	56.4	500	94.0	467	170				
Methyl pheophorbide *a*	630	27.5	580	66.5	546	56.0	522	26.2	481	30.8	452	39.8		
10-oxy-methyl pheophorbide *a*	630	29.8	582	82.7	546	61.3	522	26.0	478	31.0	450	33.3		
10-methoxy-methyl pheophorbide *a*	630	28.6	582	81.6	544	83.4	521	29.0	480	32.3	454	37.3		

the color reverts to blue-green. Weller (35) found that the intermediate is stable in the absence of oxygen and measured its absorption spectrum in pyridine containing a small amount of methanolic KOH.

The following convenient variations of the phase test were made using *ca.* $10^{-4}M$ solutions (1 to 4 ml.) of pigment:

Solvent	Reagents
pyridine	10% aqueous KOH (0.1 ml.) methanolic magnesium methoxide (0.5 ml.)
dimethylformamide	0.5% methanolic KOH (0.05 ml.) 10% aqueous KOH (0.1 ml.) methanolic magnesium methoxide (0.5 ml.)
ether, acetone, or benzene	10% methanolic KOH (0.01 ml.)

Aqueous KOH added to an acetone solution did not generate the intermediate of chlorophyll *a* but did generate that of pheophytin *a*. To be noted is the fact that it was not necessary that methanol be present or water be absent for the intermediate to appear.

The color and the absorption spectrum of the intermediate in pyridine (or dimethylformamide) depended on the base used. In the presence of methanolic KOH the color was red-brown and the spectrum was identical with that given by Weller (35). Most noticeable was an absorption maximum at 525 mμ which was more intense than either of the "blue" or "red" maxima.

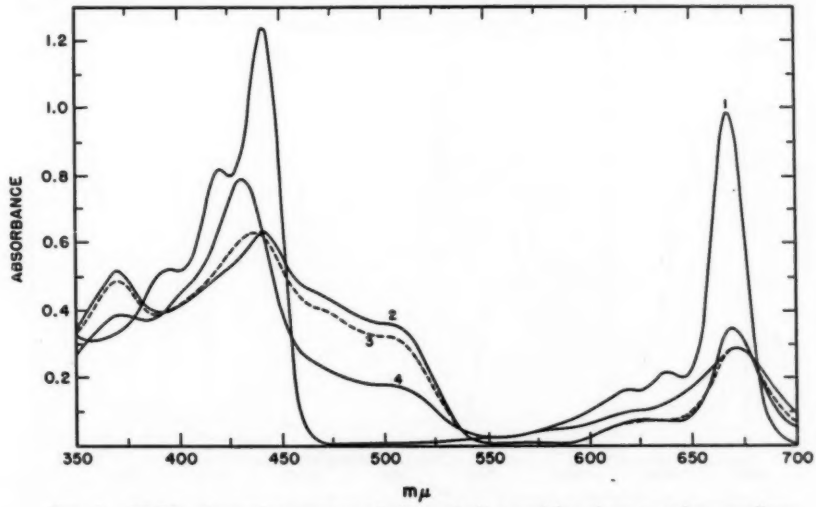


FIG. 2. Visible absorption spectra of chlorophyll *a* and its phase test intermediate. (1) chlorophyll *a* (1.15×10^{-5} *M*) in pyridine. (2), (3), (4) : the phase test intermediate 15 seconds, 350 seconds, and 50 minutes respectively, following the addition of 0.2 ml. of methanolic magnesium methoxide to 4 ml. of the pyridine solution. Final magnesium concentration : 0.9 mg./ml.

In addition, the "red" maximum relative to that of chlorophyll *a* was shifted 17 mμ toward the red region of the spectrum. In pyridine containing methanolic magnesium methoxide the intermediate was yellow-green and was quite stable even under aerobic conditions. Fig. 2, Curve 2, shows that there was a general absorption region from 455 to 540 mμ, with a hint of a maximum at 505 mμ. In the red and blue portions of the spectrum, absorption had decreased without pronounced shifts of the maxima. This might or might not indicate incomplete conversion into the phase test intermediate. The spectrum is similar to that of the intermediate in ether containing trimethylbenzylammonium hydroxide, measured by Dunicz *et al.* (6).

In Fig. 3 are absorption spectra from dimethylformamide solutions of pheophytin *a* when (*a*) methanolic magnesium methoxide was added under aerobic conditions, and (*b*) methanolic KOH was added under anaerobic conditions. Decomposition was noted in approximately 30 minutes in the test with magnesium methoxide, but this was found also in the absence of magnesium methoxide. (When pyridine was the solvent the spectrum of the intermediate changed only slightly during 4 hours; intermediate still remained after 24 hours.) The absorption maxima in dimethylformamide–KOH were at 373, 523, 661, and 711 mμ; those in dimethylformamide – magnesium methoxide were at 364, 385, 437, 462, 493, 640, and 691 mμ, and those in methanolic magnesium methoxide (given in the order of their decreasing

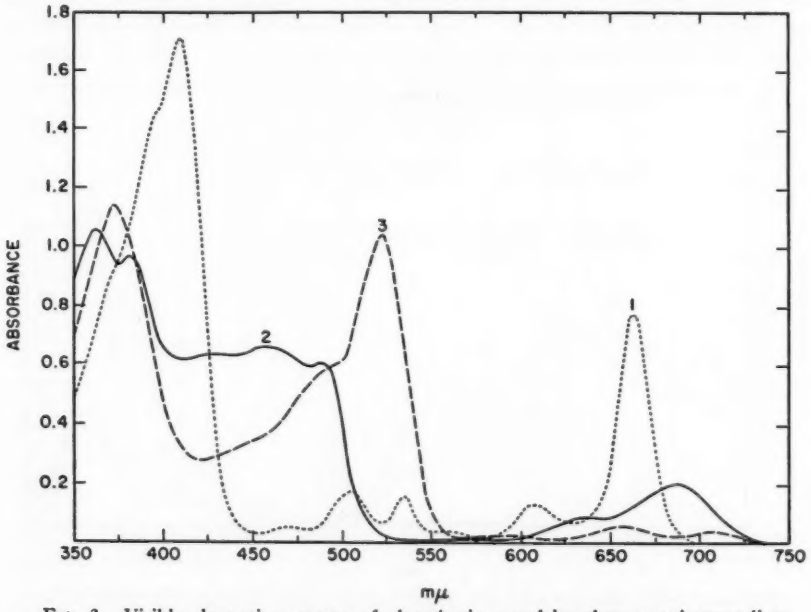


FIG. 3. Visible absorption spectra of pheophytin *a* and its phase test intermediate. (1) pheophytin *a* (1.66×10^{-5} *M*); (2) 30 seconds after the addition of 0.05 ml. of methanolic magnesium methoxide (18 mg. Mg/ml.) to an aerobic solution; (3) 30 seconds after the addition of 0.05 ml. of methanolic potassium hydroxide (10%, w/v) to an anaerobic solution. Solvent: dimethylformamide. Initial volume: 5 ml.

absorbance) were at 359, 426, 385, and 685 mμ, with slight shoulders at *ca.* 463 and 490 mμ. When air was admitted to the solution containing KOH the color became blue-green, and a test sample transferred to ether and dried no longer gave a positive phase test. In ether containing a small amount of methanolic KOH and kept under anaerobic conditions the intermediate appeared straw-yellow. The maximum at 504 mμ and the minimum at *ca.* 440 mμ were less pronounced than the corresponding maximum and minimum observed in dimethylformamide–KOH, but far more pronounced than those in dimethylformamide – magnesium methoxide.

Formation of the intermediate in sufficient concentration to be visible to the eye was influenced by the acidity of the solvent. In methanol the intermediate of chlorophyll *a* was not observed until the concentration of KOH reached *ca.* 15%. In tertiary butanol, which is far less acidic than methanol (22), the intermediate was observable in the presence of 0.5% KOH.

Under aerobic conditions 0.5% methanolic KOH converted methyl chlorophyllide *a* chiefly into Mg-chlorin e_6-trimethyl ester whose visible absorption spectrum is given in Fig. 9. This product was identified from the spectrum of its magnesium-free derivative (31) and from the fact that the same derivative was converted into the phase test intermediate of pheophorbide *a* by addition of an equal volume of 10% methanolic KOH to a pyridine solution (12). In more concentrated methanolic KOH solutions (5% to 30%) Mg-chlorin e_6 products were formed only when oxygen was excluded, e.g., by heating of the solutions to boiling. The above results appear to indicate that in dilute methanolic KOH the concentration of intermediate was so small that even under aerobic conditions methanolysis could proceed far faster than oxidation.

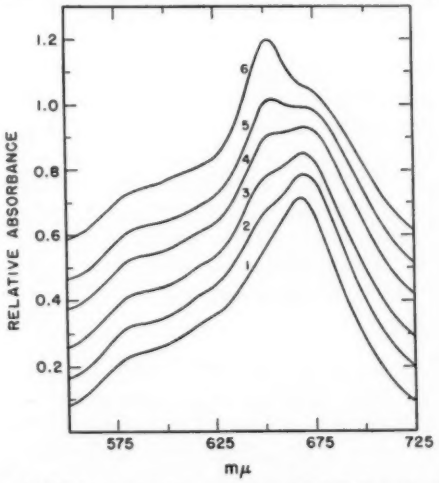


FIG. 4. Change of visible absorption spectrum (550–725 mμ) of oxidation product of chlorophyll *a*. Solvent : methanolic magnesium methoxide (18 mg. Mg/ml.) containing air. Time elapsed after addition of solvent to pigment: (1) 30 seconds; cf. Fig. 6, curve 1; (2) 30 minutes; (3) 1 hour; (4) $1\frac{3}{4}$ hours; (5) $3\frac{1}{2}$ hours; (6) 24 hours. At 725 mμ each curve was raised *ca.* 0.1 absorbance unit above the preceding curve.

In contrast to methanolic KOH the stock solution of methanolic magnesium methoxide did not produce Mg-chlorin e_6-trimethyl ester under anaerobic conditions even after it was boiled for 30 minutes. Only the phase test intermediate was formed, which upon addition of a mixture of an aqueous solution of KH_2PO_4 and ether reverted to methyl chlorophyllide *a*. Under aerobic conditions considerable changes occurred as seen from Figs. 4 and 5. These are referred to in more detail below.

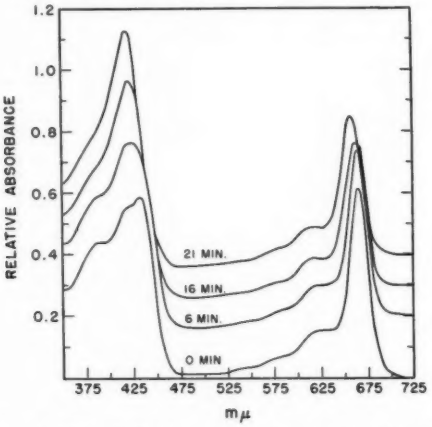


FIG. 5. Oxidation of chlorophyll *a* in untreated reagent methanol containing magnesium methoxide and air. Initial chlorophyll concentration: 0.83×10^{-5} *M*. Magnesium concentration: 2.5×10^{-3} mg./ml. At 725 mμ the upper three curves were raised above the abscissa 0.2, 0.3, and 0.4 absorbance unit respectively.

2. Allomerization in Methanol

Methyl chlorophyllide *a* (100 mg.) in dried methanol (200 ml.) saturated with oxygen was let stand in darkness in a loosely stoppered flask for 12 hours. A test sample no longer gave the phase test intermediate. The pigment was transferred to ethyl ether and after being dried was adsorbed on a sugar column. Isopropanol (0.7%) in petroleum ether resolved the pigment into three zones. These, in the order of decreasing rate of movement down the column, are referred to as fractions 1, 2, and 3. Each fraction was chromatographed twice more. The visible absorption spectra of ethyl ether solutions are given in Fig. 6; infrared absorption spectra of the corresponding phytyl esters are given in Fig. 7.

(a) Fraction 1: Mg-purpurin 7-trimethyl ester

The acid number, 11–12, and the visible absorption spectrum of the magnesium-free derivative matched those of purpurin 7-trimethyl ester (5, 32). Infrared absorption spectra supported this assumption. For comparison, Mg-purpurin 7-trimethyl ester was prepared by reacting Mg-"unstable" chlorin monoester with diazomethane (5,13). Methyl chlorophyllide *a* (15 mg.) was dissolved in pyridine (2 ml.) saturated with oxygen and aqueous 10% KOH (0.2 ml.) was added. This caused appearance of the phase test

intermediate, which disappeared after 1 to 2 minutes. The solution was added to a separatory funnel containing ether (150 ml.) and aqueous 5% KH_2PO_4 (200 ml.). After several washings with acid phosphate, and a final wash with distilled water, the ether solution of pigment was added to an ether solution of diazomethane. After 15 minutes the sharp red absorption band at 650 mμ changed to give a broad diffuse band with a maximum at 670 mμ. The product was purified by chromatography. Its acid number was 12, and its visible and infrared absorption spectra matched those of fraction 1. Both this product and fraction 1 were converted into Mg-"unstable" chlorin by 30% methanolic KOH (19).

A convenient method of preparation which avoided producing "unstable" chlorin and treating the latter with diazomethane was oxidation of the phase test intermediate by oxygen in the stock methanolic magnesium methoxide solution. The oxidation was extremely rapid. The spectrum of the product from 550 mμ to 725 mμ 30 seconds after addition of the pigment to the base is given in Fig. 4, Curve 1. Fraction 1 was gradually converted into a product whose absorption maximum was at *ca.* 652 mμ, i.e., into fraction 2 (Figs. 5 and 6). Hot, dilute methanolic KOH also converted fraction 1 into fraction 2.

(b) Fraction 2: Mg-purpurin 7-lactone-methyl ether-dimethyl ester

In common with the product isolated by Fischer and Pfeiffer (14) this fraction had the following characteristics: (1) the visible absorption spectrum of the magnesium-free derivative matched closely that of "unstable" chlorin-monomethyl ester (30); (2) it was not extracted by aqueous 10% KOH from

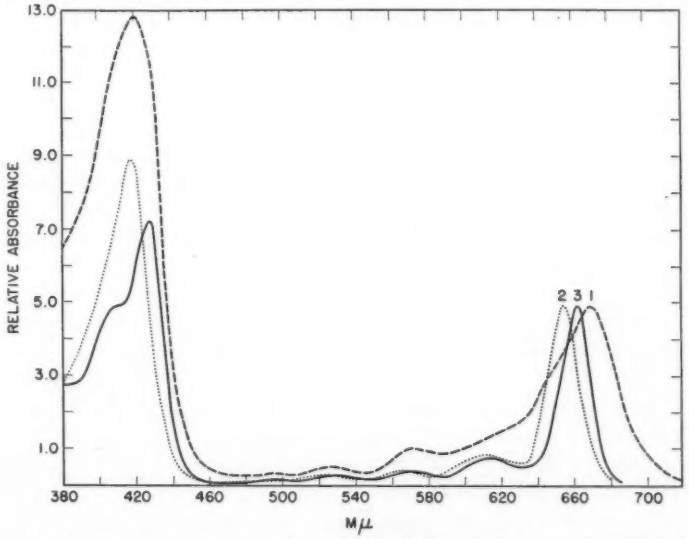


FIG. 6. Visible absorption spectra of allomerization derivatives of methyl chlorophyllide *a* in ethyl ether. (1) fraction 1 (Mg-purpurin 7-trimethyl ester); (2) fraction 2 (Mg-purpurin 7-lactone-methyl ether-dimethyl ester); (3) fraction 3 (Mg-10-oxy-methyl pheophorbide *a*). Each curve was adjusted to make the "red" absorption maxima equal.

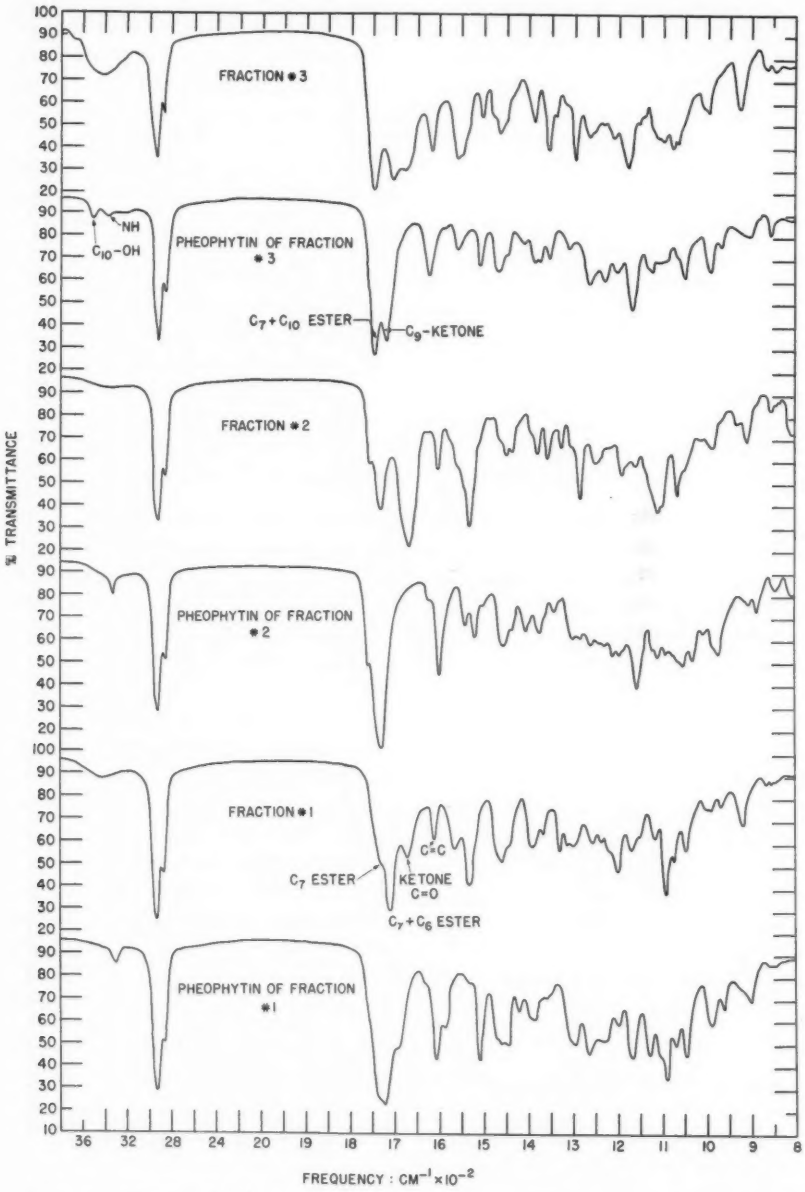


FIG. 7. Infrared absorption spectra of the phytyl esters corresponding to fractions 1, 2, and 3 and their magnesium-free derivatives. Solvent: CCl_4. Concentration: 5–6% (w/v).

ether solution; (3) it did not react with diazomethane to give fraction 1; (4) the methoxyl content of the magnesium-free derivative indicated three methoxyl groups (found: 13.9%; calculated: 14.4%). The acid number was 15. When this derivative was dried and stored in a test tube it formed Mg-purpurin 18-methyl ester.

Fraction 2 was prepared conveniently by exposing methyl chlorophyllide *a* to a dilute solution of magnesium methoxide in untreated reagent methanol containing oxygen (see Fig. 5). The concentration of magnesium methoxide had to be adjusted to permit following the spectral changes with time; at higher concentrations the reaction was too rapid.

(c) Fraction 3: Mg-10-oxy-methyl Pheophorbide a

Evidence for the cyclopentanone ring was (1) the presence in the infrared absorption spectrum of a ketone absorption band at 1691 cm.$^{-1}$ (25); (2) the relatively high acid number, 15, since absence of a ring structure between C_γ and C_6 usually lowers the acid number below 10 (13); the close similarity of the visible absorption spectrum of this compound and its derivatives to those of methyl chlorophyllide *a* and its corresponding derivatives. The derivatives were the magnesium-free compounds and their 2,4-dinitrophenylhydrazones (24); sodium borohydride reduction products of the phyllins (23); and aminolysis products from treatment of the phyllins with ethanolamine (8).

Evidence for the C_{10} carbomethoxy group was the presence of an ester absorption band in the infrared spectrum of the free acid of the magnesium-free derivative, and the methoxyl content of the free acid: 9.34%. Calculated: 10.6%.

Evidence of the C_{10} hydroxyl group was (1) the presence of a hydroxyl absorption band at 3520 cm.$^{-1}$ in the infrared spectrum of the magnesium-free derivative; (2) the fact that extraction from an ether solution into 10% aqueous KOH by shaking for 1–2 hours converted fraction 3 into Mg-"unstable" chlorin-monomethyl ester and small amounts of Mg-purpurin 18. The formation of these products was demonstrated spectrophotometrically (Fig. 9), and by the reaction of Mg-"unstable" chlorin-monomethyl ester with diazomethane to produce Mg-purpurin 7-trimethyl ester. These results agreed with those obtained with 10-oxy-methyl pheophorbide *a* under the same conditions (10).

Hot 0.2% methanolic KOH converted fraction 3 into fraction 2; stock methanolic magnesium methoxide solution did likewise at room temperature but more slowly.

A different method of preparation of this compound is as follows: to pyridine (75 ml.) containing *p*-benzoquinone (1.0 g.) and methyl chlorophyllide *a* (40 mg.) was added aqueous *N*/10 NaOH (10 ml.). The mixture (which immediately became black owing to decomposition of quinone) was shaken for 2 minutes and poured into a separatory funnel containing ethyl ether (400 ml.). Quinone and its decomposition products were removed by thorough washings with dilute NH_4OH. A test sample of the pigment gave a weak phase test. The pigment was chromatographed and contained, in addition

to the desired product, unchanged methyl chlorophyllide *a*, material which precipitated from 4% pyridine in petroleum ether, and an unknown phyllin whose "red" absorption maximum was at *ca.* 667 mμ. The yield was 50%. An advantage of this method was that preparation of the 10-acetoxy derivative and its subsequent acid hydrolysis to the 10-oxy compound was eliminated (9,10).

Addition of quinone to the phase test intermediate gave much poorer yields than addition of alkali to the pigment–quinone solution. When iodine was used in place of quinone, the product had the spectrum of fraction 2.

(d) Estimation of the Relative Amounts of Fractions 1, 2, and 3

The amounts of fractions 1 and 2 were determined in dioxane solutions of the magnesium-free derivatives using known molar extinction coefficients (30, 32). Because the extinction coefficient of the 10-oxy derivative was not available, the concentration of this product was estimated by converting it into fraction 2 in methanolic magnesium methoxide. Each of fractions 1 and 3 accounted for 10–15% of the total allomerized pigment. From these figures it was calculated that the ratio of oxygen consumed to methyl chlorophyllide *a* allomerized was 0.92. This figure agrees quite well with those found earlier, which varied from 0.93 to 1.08 (13).

Subsequent experiments showed that the relative amounts of fractions 1 and 2 could be changed by adding magnesium methoxide to methanol, higher concentrations favoring formation of fraction 1, as can be seen from Figs. 4 and 5.

3. Preparation of Other Allomerized Derivatives

(a) Mg-10-methoxy-methyl Pheophorbide a

The corresponding ethoxy derivative has been prepared by exposing methyl chlorophyllide *a* to oxygen-free ethanol containing *p*-benzoquinone (15, 17). When iodine was used in place of quinone the magnesium-free 10-ethoxy compound resulted.

A different method is as follows: methyl chlorophyllide *a* (30 mg.) in pyridine (10 ml.) and methanolic magnesium methoxide (100 ml.) were flushed separately with nitrogen for 10 minutes. The solutions were mixed, causing appearance of the phase test intermediate, and 0.1 *M* iodine (10 ml.) was added immediately. On the addition of iodine, the original blue-green color returned. The mixture was poured into a separatory funnel containing ethyl ether (300 ml.), hydrochloric acid (2% w/v, 300 ml.), and ascorbic acid (0.3 g.). By vigorous shaking magnesium hydroxide was completely dissolved, and the ether solution was then washed several times with distilled water. The solution was dried *in vacuo* and the pigment was purified chromatographically by treatment with benzene, and with isopropanol – petroleum ether mixtures (0.7 to 1.4%). The yield was 40%.

That this product was the 10-methoxy derivative could be deduced from results similar to those found for fraction 3 with the exception that the methoxyl content of the free acid of the magnesium-free derivative was 9.4%.

Calculated: 10.6%. In addition, no hydroxyl absorption band was present in the infrared absorption spectrum of the magnesium-free derivative. Infrared spectra of the phytyl derivatives are given in Fig. 8.

Readily observable differences between this compound and the 10-oxy derivative were (1) the ratio of the absorbance of the "blue" maximum to that of the "red" maximum was 1.44 for the 10-oxy derivative and 1.26 for the 10-methoxy derivative; (2) whereas 30% methanolic KOH converted the 10-oxy derivative to Mg-"unstable" chlorin, it converted the 10-methoxy derivative into a product having a visible absorption spectrum similar to that of Mg-chlorin e_6-trimethyl ester. The product was probably Mg-rhodochlorin (19).

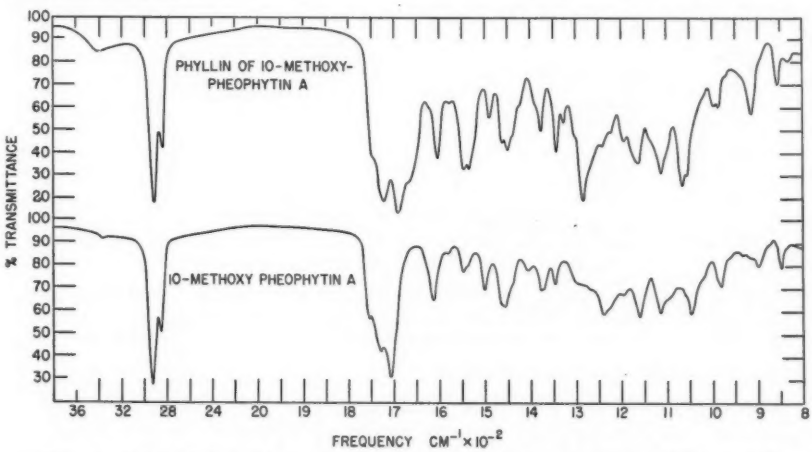


FIG. 8. Infrared absorption spectra of the phyllin of 10-methoxy-pheophytin *a* and its magnesium-free derivative. Solvent: CCl_4. Concentration: 5% (w/v).

(b) Mg-"unstable" chlorin-methyl-phytyl ester

This compound can be prepared by oxidation of the phase test intermediate by oxygen in pyridine containing aqueous KOH (13). A disadvantage of the method is that hydrolysis of the C_7 ester reduces the yield considerably. Fischer and Kahr (11) used permanganate in pyridine to convert pheophorbide *a* into "unstable" chlorin-monomethyl ester.

The phyllin of the diester was prepared by oxidizing the phase test intermediate of chlorophyll *a*, generated in pyridine by aqueous KOH, with a 1% solution of potassium permanganate in acetone, and by transferring the pigment into ethyl ether immediately. The yield was 50%.

Visible and infrared absorption spectra are given in Figs. 9 and 10. The visible spectrum is seen to be very similar to that of fraction 2. The infrared spectrum of the magnesium-free derivative shows the expected hydroxyl band (Fig. 10).

The dimethyl ester is totally extracted from ether solution by aqueous 10% KOH; its acid number is 12.

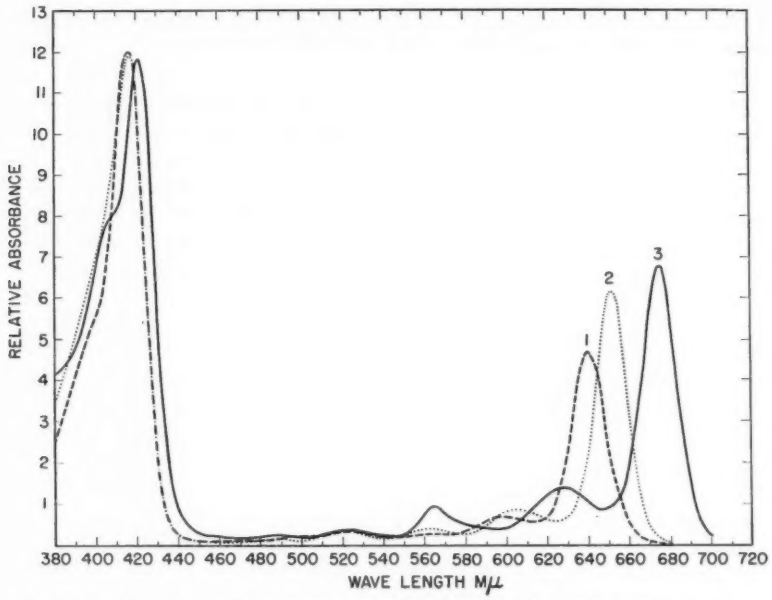


FIG. 9. Visible absorption spectra of the phyllins of (1) chlorin e_6-trimethyl ester; (2) "unstable" chlorin-methyl-phytyl ester; (3) purpurin 18-methyl ester. Solvent—ethyl ether. Each curve was adjusted to make the "blue" absorption maxima equal.

(c) Mg-purpurin 18-methyl ester

This compound was always isolated from columns during purification of "unstable" chlorin-dimethyl ester prepared by oxidation of the phase test intermediate with oxygen. It was readily identified from the absorption spectrum of the magnesium-free derivative, which had a "red" absorption maximum at 698 mμ, from its high acid number, 20, and from its ready conversion to chlorin p_6 (5, 13). Its visible and infrared absorption spectra are given in Figs. 9 and 10 respectively.

Comments on the Visible Absorption Spectra

From Table I it is seen that replacement of the H atom at C_{10} of chlorophyll *a* by either an OH or OCH_3 group affects the absorption spectrum only very slightly. Similarly, removal of the C_{10}-carbomethoxy to form Mg-methyl pyropheophorbide *a* affects the spectrum only slightly (23). The spectra of all the other allomerization derivatives are markedly different from that of chlorophyll *a*. The transition from the broadened maxima of fraction 1 to the sharp ones of fraction 2 is analogous to that which occurs between their corresponding pheoporphyrin derivatives (10).

The wavelength of the "red" maximum of Mg-chlorin e_6-trimethyl ester does not agree with that given earlier (34), being 641 mμ (Table I) instead of 650 mμ. Another discrepancy is the "red" maximum of 10-ethoxy-methyl pheophorbide *a* is reported to be at 663 mμ and that of 10-ethoxy-ethyl

pheophorbide *a* to be at 667.5 mμ (15, 17). Since substitution of the C_7 phytyl ester by an ethyl or methyl group does not cause any spectral shift of the maximum (24) one of the two wavelengths must be incorrect. We believe the higher figure to be the correct one since the "red" maxima of 10-oxy, 10-methoxy, and pyropheophorbide *a* are very close to that of pheophorbide *a*, 667 mμ.

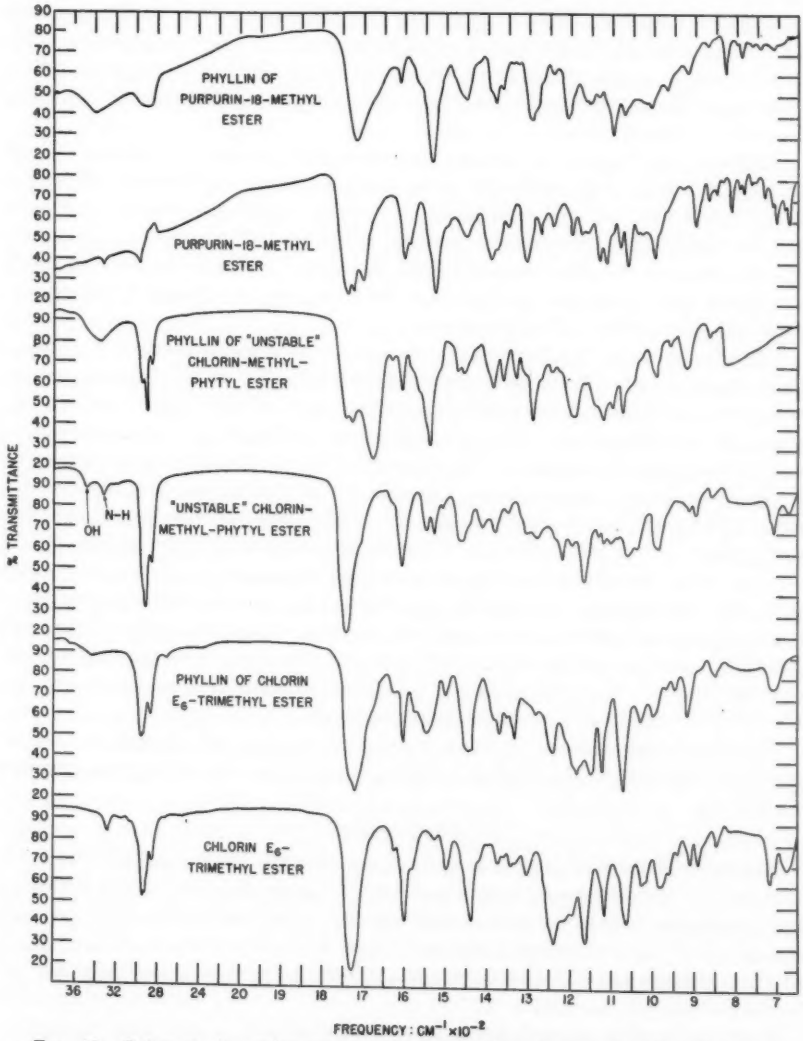

PHYLLIN OF PURPURIN-18-METHYL ESTER
PURPURIN-18-METHYL ESTER
PHYLLIN OF "UNSTABLE" CHLORIN-METHYL-PHYTYL ESTER
"UNSTABLE" CHLORIN-METHYL-PHYTYL ESTER
OH
N-H
PHYLLIN OF CHLORIN E_6-TRIMETHYL ESTER
CHLORIN E_6-TRIMETHYL ESTER
% TRANSMITTANCE
FREQUENCY: $CM^{-1} \times 10^{-2}$
36 32 28 24 20 19 18 17 16 15 14 13 12 11 10 9 8 7

FIG. 10. Infrared absorption spectra of the phyllins of purpurin 18-methyl ester, "unstable" chlorin-methyl-phytyl ester, and chlorin e_6-trimethyl ester and their magnesium-free derivatives. The upper two curves were determined from Nujol mulls; the remainder from CCl_4 solutions. Concentration: 5–6% (w/v).

Comments on the Infrared Absorption Spectra

The spectra of the phyllins of chlorophyll derivatives in Figs. 7, 8, and 10 are more difficult to reconcile with their given chemical structures than are the spectra of chlorophylls *a* and *b*. This is especially so in the double bond stretching region. The spectra of fraction 2 and of Mg-"unstable" chlorin-methyl-phytyl ester (Figs. 7 and 10) are not consistent with those to be expected for six-membered ring lactols (2, 20); nor is that of Mg-purpurin 18-methyl ester characteristic of an anhydride (1). Those of fraction 1 and of Mg-chlorin e_6-trimethyl ester are consistent with their structures. Both possess an absorption band at *ca.* 1720 cm.$^{-1}$ which we assign to the conjugated C_6 carbomethoxy group.

Removal of magnesium causes a considerable number of changes in the spectra. A most noticeable one is the shift of the intense band at 1670 cm.$^{-1}$ in the spectra of fraction 2 and of Mg-"unstable" chlorin diester to 1735 cm.$^{-1}$; the resulting spectra are not inconsistent with those of lactols (2). One could assume that on removal of magnesium the keto derivative is converted into the lactol were it not for the fact that the spectrum of fraction 1 (keto form) does not agree with that of fraction 2.

Equally puzzling is the band at 1670 cm.$^{-1}$ in the spectrum of fraction 3 (Fig. 7) and of Mg-10-methoxy-pheophytin *a* (Fig. 8), which disappears when magnesium is removed. This band is reminiscent of that which occurs in the spectrum of chlorophyll *a* (25) and which was attributed to hydrogen bonding of the enol of the β keto ester. Enolization of the C_9 keto group is not possible in the allomerized derivatives. The spectra of the 10-oxy and 10-methoxy magnesium compounds are very similar while those of the magnesium-free derivatives are quite different. The keto group of 10-oxy-pheophytin *a* absorbs more intensely than the ester groups whereas the reverse is the case with the 10-methoxy compound. Furthermore, the 10-methoxy compound possesses an additional sharp band on the high frequency side of the ester band. (A similar difference is noted between the spectra of the magnesium-free derivatives of fraction 2 and "unstable" chlorin diester, which also are 10-methoxy and 10-oxy compounds respectively.)

The above observations again emphasize the marked influence of the electropositive magnesium atom upon carbonyl groups in ring structures between C_6 and C_7.

Discussion

Fischer and Pfeiffer (14) have postulated that the first product of reaction between methyl chlorophyllide *a* and oxygen in methanol or ethanol is a C_{10} hydroperoxide, whose formation labilizes the cyclopentanone ring and causes the latter to split hydrolytically and place a transient free carboxyl group at C_6. Subsequent reaction with the solvent and lactonization produces the lactone ether, i.e. fraction 2.

From the results presented here it is evident that it is the phase test intermediate which reacts with oxygen. The concentration of the intermediate depends on the concentration of alkali; the higher the concentration of alkali the faster is the rate of allomerization.

The mechanism of Fischer and Pfeiffer does not account for the formation of fraction 1 which possesses a C_6 carbomethoxy group. Fraction 1 was the predominant initial product when allomerization was carried out in stock methanolic magnesium methoxide (Fig. 4), while fraction 2 was the predominant product when allomerization was carried out in extremely dilute methanolic magnesium methoxide (Fig. 5). A significant difference between the two conditions was that the dilute solution was not anhydrous and thus contained hydroxyl ions, while the concentrated solution was anhydrous and contained only methoxide ions. Fraction 1 apparently results from addition of methoxide ion to the C_9 carbonyl group followed by ring splitting and simultaneous loss of a hydroxyl ion from the C_{10} hydroperoxide group. On the other hand, formation of fraction 2 appears to result when hydroxyl ions are present. The group eventually attached to C_{10} of the lactone will depend on solvation of the C_7 alpha keto acid. Fraction 2, which contains a C_{10} methoxy group, results when the solvent is methanol, while Mg-"unstable" chlorin ester, which contains a C_{10} hydroxyl group, results when the solvating group is water, e.g. when aqueous KOH is added to a pyridine solution of methyl chlorophyllide *a*. These conclusions are supported by the fact that neither fraction 1, nor fraction 3, nor Mg-"unstable" chlorin ester are the direct precursors of fraction 2. These findings were shown by the following: (1) no fraction 2 was formed when these compounds were permitted to remain up to 48 hours in samples of the methanol used in the allomerization experiments; (2) each of the compounds was stable in methanolic solutions of methyl chlorophyllide *a* undergoing allomerization (the amount recovered equalled the amount added plus the amount formed in a control experiment, with the exception that no Mg-"unstable" chlorin ester was formed in methanol). The fact that allomerization in redistilled methanol produced some Mg-purpurin 7-trimethyl ester (fraction 1) suggests that magnesium methoxide or some magnesium compound distilled over with methanol itself. Formation of fraction 3, which is also not accounted for by the hypothesis of Fischer and Pfeiffer, can result from dismutation of the hydroperoxide or its reduction by a molecule of phase test intermediate.

In the oxidation by quinone, formation of a hydroquinone ether and its subsequent hydrolysis or alcoholysis has been postulated (18). The hydroquinone ether was not demonstrated directly. Examination of infrared spectra of chlorophyll *a* in methanol containing various concentrations of quinone failed to reveal definite evidence of a chlorophyll–quinone complex. Subsequent investigation should reveal if the phyllin whose "red" absorption maximum is at 676 mμ is a new allomerization derivative containing quinone. Oxidation by iodine to produce the C_{10}-methoxy derivative can be explained by substitution of iodine for the C_{10} hydrogen and its displacement by a methoxide ion (21).

It is puzzling that the absorption spectrum of the phase test intermediate in pyridine (or dimethylformamide) is dependent upon the base added. It is possible that when methanolic KOH is added, a much higher concentration of methoxide ion (or hydroxide ion) is present than when methanolic

magnesium methoxide is added. This explanation may account for the marked instability of the intermediate of pheophytin *a* toward oxygen when methanolic KOH is added. Of some relevance may also be the fact that in a salt of the enolate ion of pheophytin *a* and $Mg(OCH_3)^+$, magnesium may be chelated by the oxygen atom of the C_{10} ester carbonyl group, whereas potassium in the potassium salt cannot be chelated. The absence of allomerization of pheophytin *a* in a dilute solution of methanolic magnesium methoxide is reminiscent of the earlier finding that this compound is not allomerized in alcohols (13).

Acknowledgments

This investigation was initiated while the author was Research Associate in the Photosynthesis Laboratory, Botany Department, University of Illinois, Urbana, Illinois, U.S.A. The author is grateful for helpful discussions with Drs. S. F. MacDonald and O. E. Edwards of the Division of Pure Chemistry, National Research Council. Infrared absorption spectra were measured by Mr. F. Rollin. Technical assistance was provided by Mrs. G. Kennedy and Mr. W. Meath.

References

1. Bellamy, L. J. The infra-red spectra of complex molecules. Methuen & Co. Ltd., London. 1954.
2. Blair, J. and Newbold, G. T. J. Chem. Soc. 2871 (1955).
3. Clark, E. P. J. Assoc. Offic. Agr. Chemists, **15**, 136 (1932).
4. Conant, J. B., Dietz, E. M., Bailey, C. F., and Kamerling, S. E. J. Am. Chem. Soc. **53**, 2382 (1931).
5. Conant, J. B. and Moyer, W. W. J. Am. Chem. Soc. **52**, 3013 (1930).
6. Dunicz, B., Thomas, T., Van Pee, M., and Livingston, R. J. Am. Chem. Soc. **73**, 3388 (1951).
7. Fischer, H., Filser, L., and Plötz, E. Ann. **495**, 1 (1932).
8. Fischer, H. and Goebel, S. Ann. **524**, 269 (1936).
9. Fischer, H. and Heckmaier, J. Ann. **508**, 250 (1934).
10. Fischer, H., Heckmaier, J., and Scherer, T. Ann. **510**, 169 (1934).
11. Fischer, H. and Kahr, K. Ann. **531**, 209 (1937).
12. Fischer, H. and Lautsch, W. Ann. **528**, 265 (1937).
13. Fischer, H. and Orth, H. Chemie des pyrrols. Vol. II, Pt. 2. Leipzig. 1940. p. 2.
14. Fischer, H. and Pfeiffer, H. Ann. **555**, 94 (1944).
15. Fischer, H. and Riedmair, H. Ann. **506**, 107 (1933).
16. Fischer, H. and Spielberger, G. Ann. **510**, 156 (1934).
17. Fischer, H. and Spielberger, G. Ann. **515**, 130 (1935).
18. Fischer, H. and Strell, M. FIAT Rev. Ger. Sci. Biochem. **I**, 142 (1947).
19. Fischer, H., Süs, O., and Klebs, G. Ann. **490**, 38 (1931).
20. Grove, J. F. and Willis, H. A. J. Chem. Soc. 877 (1951).
21. Hine, J. Physical organic chemistry. McGraw-Hill Book Co., Inc., New York. 1956
22. Hine, J. and Hine, M. J. Am. Chem. Soc. **74**, 5266 (1952).
23. Holt, A. S. Unpublished results.
24. Holt, A. S. and Jacobs, E. E. Am. J. Botany, **41**, 710 (1954).
25. Holt, A. S. and Jacobs, E. E. Plant Physiol. **30**, 553 (1955).
26. Jacobs, E. E., Vatter, A., and Holt, A. S. Arch. Biochem. Biophys. **53**, 228 (1954).
27. Kolthoff, I. M. and Sandell, E. B. Quantitative inorganic analysis. The Macmillan Co., New York. 1946.
28. Molisch, H. Ber. deut. botan. Ges. **14**, 16 (1896).
29. Rabinowitch, E. I. Photosynthesis. Vol. II, Pt. 2. Interscience Publishers, Inc., New York. 1956. p. 2.
30. Stern, A. and Pruckner, F. Z. physik. Chem. (Leipzig) A, **180**, 321 (1931).
31. Stern, A. and Wenderlein, H. Z. physik. Chem. (Leipzig) A, **174**, 81 (1935).
32. Stern, A. and Wenderlein, H. Z. physik. Chem. (Leipzig) A, **176**, 81 (1936).
33. Strain, H. H. J. Agr. Food Chem. **2**, 1222 (1954).
34. Treibs, A. and Wiedemann, E. Ann. **471**, 146 (1929).
35. Weller, A. J. Am. Chem. Soc. **76**, 5819 (1954).

METABOLIC CHANGES IN TRAUMA-RESISTANT RATS FOLLOWING TRAUMA[1]

F. Chytil and Z. Hruza

Abstract

Some metabolites were followed in rats which had been made resistant to trauma by exposure to Noble–Collip drum injury. In trauma-resistant rats liver glycogen levels are 50% lower than in control animals. If trauma-resistant rats are subjected to trauma that does not kill control animals then the metabolic response during the first 2 hours after trauma, determined by measuring changes in blood glucose, pyruvic acid, amino nitrogen, urea, and total acetone bodies, differs from that of control animals only by being less pronounced quantitatively. The course of metabolic changes in trauma-resistant rats differs considerably from that of control animals between the 2nd and 24th hour after trauma, especially in the levels of acetone bodies and urea in the blood. The development of the resistance to trauma is accompanied by a change in the metabolic response to trauma.

Introduction

Trauma-resistant rats can survive trauma that is several times the lethal dose for control animals (12). Neufeld *et al.* (11), McShan *et al.* (9), Le Page (7), and recently Manning and Hampton (8) studied biochemical changes in trauma-resistant animals. It is not yet known whether during the development of resistance an adaptation of metabolism also occurs.

In this paper it was attempted to determine whether trauma-resistant animals differ in their metabolic response to trauma from those exposed to trauma for the first time. The levels of some metabolites were followed in the blood and some tissues in trauma-resistant and control animals together with the course of metabolic changes during the first 24 hours following trauma.

Experimental

Development of Resistance

Adult male or female rats of the Wistar strain were fed on a standard diet which contained 52.8% carbohydrate, 25.5% protein, and 21.7% fat (calculated in caloric values). Animals were subjected to increasing amounts of trauma in a Noble–Collip drum constructed according to Noble and Collip (13) and having two projections. The number of rotations was 45 per minute (the average number of falls 49 per minute). The timetable of increasing traumatization is given in Fig. 1. The initial dose of continuous trauma was 8 minutes (380 turns). During increasing traumatization lasting 23 days the body weight and food intake of the experimental animals were determined. After the last dose of trauma (30 minutes, 1350 turns), animals were kept at rest for 9 days. A dose of 1350 turns is 100% lethal in control animals but did not cause a single death in the trauma-resistant group. A group of trauma-resistant rats was then, after an interval of 9 days, traumatized for 10 minutes in the drum (450 turns). This time corresponds to a traumatizing

[1]Manuscript received November 12, 1957.
Contribution from Laboratory of Physiology and Pathophysiology of Metabolism, Czechoslovak Academy of Sciences, Prague.

energy of 460 kg./g. body weight (6). This trauma did not kill control animals that were traumatized with the same number of turns. Trauma-resistant rats and controls were usually killed in groups of six before trauma and after 0, 0.5, 1, 2, 3.5, 6, and 24 hours after a 10 minute stay in the Noble–Collip drum (450 turns). The animals were killed by decapitation; blood was collected into potassium oxalate to prevent clotting. Immediately after a blood sample had been taken, samples of liver were taken for analysis. During the whole experiment period all animals had free access to food and water.

Analytical Methods

The method of Good *et al.* (4) was used for extraction and hydrolysis of the glycogen. Glucose determinations on the glycogen hydrolyzates and on blood were carried out by the colorimetric procedure of Nelson (10). Pyruvic acid was determined in the blood using a modification of the Friedmann-Haugen colorimetric method (15). Amino nitrogen in blood was determined according to Frame *et al.* (3). Blood urea was determined colorimetrically using a diacetyl reagent (14). It was found that this method gave results identical with results obtained using the method of Conway and O'Malloy (2). Total acetone bodies in the blood were determined according to Thin and Robertson (16). All analyses were carried out in duplicate.

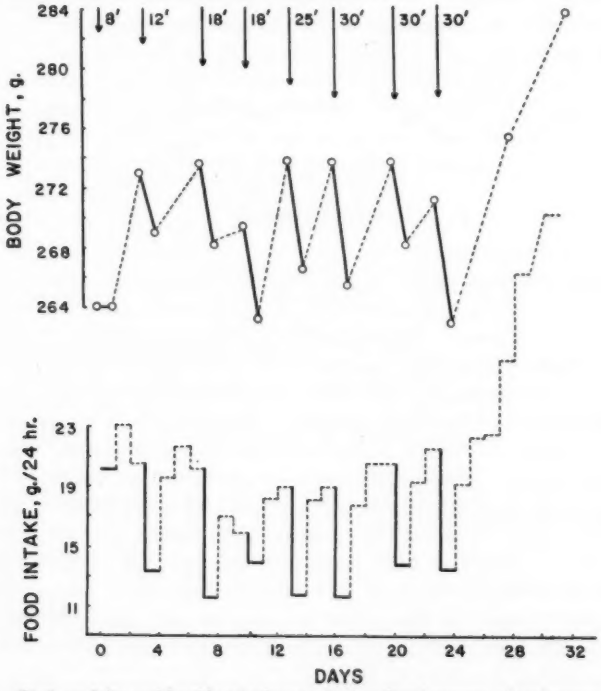


FIG. 1. Body weight and food intake in rats during development of resistance. Arrows indicate days at which animals were traumatized; the dose of trauma is given in minutes during which animals were in the Noble–Collip drum. Each value the average of 90 rats.

Results

Body Weight and Food Intake during the Development of Resistance to Trauma

During the first 24 hours after trauma rats always lost body weight and showed a decreased food intake. After applications of trauma had been stopped both food intake and weights increased suddenly (Fig. 1).

Resting levels determined in trauma-resistant rats 9 days after the last trauma are given in Table I. It can be seen that the concentration of glycogen in the liver is on the average 50% lower than in control animals. (Muscle glycogen was also decreased, but this difference was not statistically significant.) The level of blood pyruvic acid is lower in all groups of trauma-resistant animals than control rats. The concentration of total acetone bodies in the blood is insignificantly increased in trauma-resistant animals. Blood urea levels are higher in trauma-resistant animals but this increase is significant only in the one group. No differences were found in the levels of blood amino nitrogen and glucose.

TABLE I

RESTING LEVELS IN TRAUMA-RESISTANT AND NON-RESISTANT RATS

Group	Sex	Mean body wt.	No. of rats	Mean	± S.E.	Significance	Deviation from non-resistant
				Liver glycogen, %			
Non-resistant	♂	403	10	5.141	0.242	$P<0.001$	−39.5%
Resistant	♂	363	10	3.110	0.268		
Non-resistant	♂	298	19	3.31	0.468	$P<0.02$	
Resistant	♂	290	9	1.18	0.273		−64.3%
Non-resistant	♀	154	15	2.84	0.309	$P<0.05$	
Resistant	♀	197	7	1.674	0.203		−41.0%
Non-resistant	♂	258	6	4.046	0.288	$P<0.001$	−54.6%
Resistant	♂	212	7	1.833	0.535		
Non-resistant	Mean of all groups		50	3.834			−49.8%
Resistant			33	1.949			
				Blood glucose, mg. %			
Non-resistant	♂	298	5	91.8	4.87	Non signif.	−10.9%
Resistant	♂	290	5	81.6	3.98		
				Blood pyruvic acid, mg. %			
Non-resistant	♂	403	10	0.654	0.039	Non signif.	−16.5%
Resistant	♂	363	10	0.547	0.050		
Non-resistant	♂	256	5	1.261	0.142	$P<0.05$	−38.2%
Resistant	♂	197	7	0.780	0.114		
Non-resistant	♂	298	13	1.053	0.135	Non signif.	−28.0%
Resistant	♂	290	5	0.758	0.109		
				Blood amino-N, mg. %			
Non-resistant	♂	403	10	11.16	0.48	Non signif.	+2.5%
Resistant	♂	363	10	11.43	0.37		
Non-resistant	♂	258	6	10.3	0.85	Non signif.	−2.5%
Resistant	♂	212	7	10.05	0.95		
				Blood urea, mg. %			
Non-resistant	♂	298	21	51.3	1.8	$P<0.01$	+25.5%
Resistant	♂	212	12	64.4	3.3	Non signif.	+ 6.5%
Non-resistant	♂	258	6	60.3	3.1		
				Blood total acetone bodies, mg. %			
Non-resistant	♂	298	12	1.402	0.118	Non signif.	
Resistant	♂	290	5	1.83	0.183		+30.5%

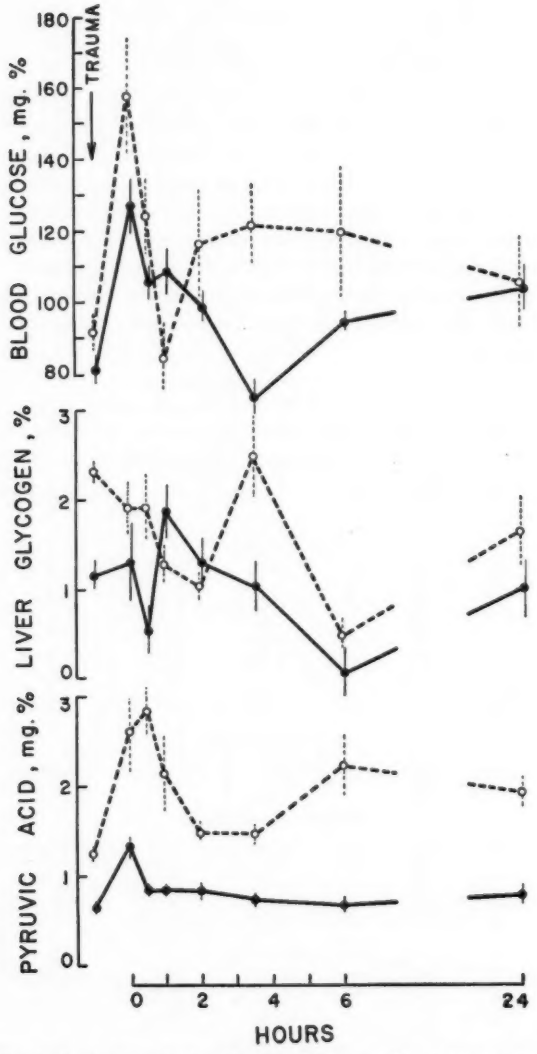


FIG. 2. Metabolic changes during the first 24 hours after injury in trauma-resistant rats (●—●) and control animals (○ - - - ○). Animals traumatized in the Noble–Collip drum (at arrow).

Abscissa: Time in hours after removal of animal from drum.

Ordinate: Levels of metabolites. Individual values are the average of at least six determinations, vertical lines at each average value indicate ± S.E.

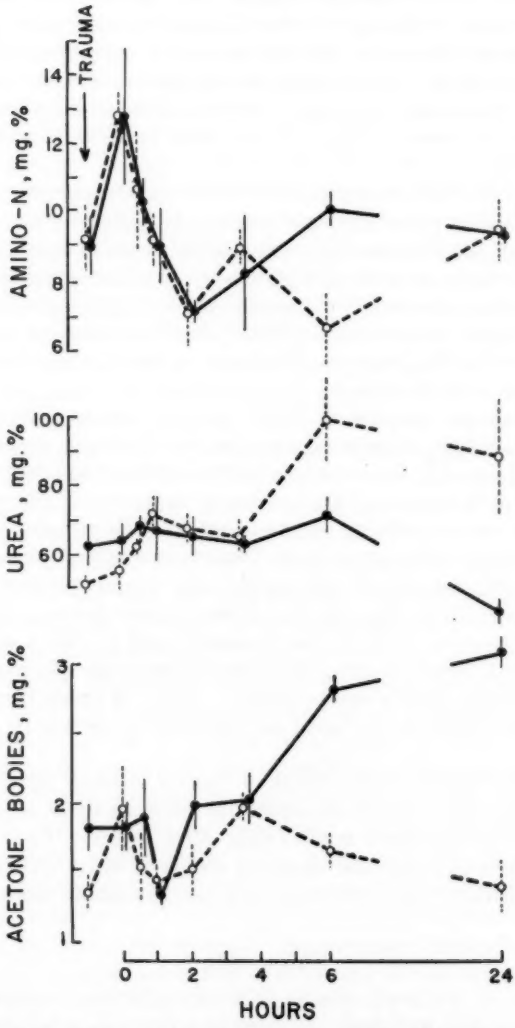


FIG. 3. Metabolic changes during the first 24 hours after injury in trauma-resistant rats (●—●) and control animals (○ - - - ○). Animals traumatized in the Noble–Collip drum (at arrow).

Abscissa: Time in hours after removal of animal from drum.

Ordinate: Levels of metabolites. Individual values are the average of at least six determinations, vertical lines at each average value indicate ± S.E.

Metabolic Changes during 24 Hours after Trauma

Figs. 2 and 3 show the changes that occur in the levels of metabolites in trauma-resistant rats and control animals after 450 turns in the Noble–Collip drum. The course of changes in blood glucose concentration is the same in both groups of animals during the first phase, i.e. roughly up to 2 hours after application of trauma. Later there are differences between the two groups. The immediate increase in glucose concentration after trauma is lower in trauma-resistant animals. The curves in both trauma-resistant and control rats are biphasic.

Changes in liver glycogen concentration also show two phases in both groups. This biphasic breakdown of glycogen is evidently typical for metabolic changes after injury and can also be observed in other types of trauma (1, 5). The decrease in glycogen concentration is slower in trauma-resistant animals and resynthesis occurs sooner. The concentration of glycogen in the liver is higher immediately after trauma (1 hour) than it was before traumatization. Glycogen stores in the liver are exhausted in both groups of experimental animals 6 hours after trauma.

In control animals changes in blood pyruvic acid concentration are also biphasic. In resistant animals, on the other hand, only an increase in pyruvic acid concentration can be observed immediately after injury; the second phase of increase is absent. There is also an indication of a biphasic response in the level of amino nitrogen in the blood in both groups. Here also the response of trauma-resistant animals differs in the second phase. Metabolic differences in the response to trauma between trauma-resistant and control animals are again found after the first 2 hours after trauma. In control rats the urea concentration in blood rises gradually and reaches a maximum after 6 hours. After 24 hours the level of urea is still elevated. In resistant animals there is no increase in urea concentration. After 24 hours there was even a decrease in urea values in the blood as compared to the resting concentration ($P < 0.05$).

Total acetone bodies in the blood of control animals again change in a typical biphasic way. In trauma-resistant animals there is no increase in acetone bodies in the blood immediately after injury. Twenty-four hours after injury the level of acetone bodies in the blood of control rats is equal to that before traumatization, whereas in trauma-resistant animals there is a large increase.

Discussion

The purpose of this work was to determine whether animals resistant to trauma differ in their metabolic response to injury—in other words, whether during development of resistance to trauma there is adaption of metabolism per se. The data concerning levels of some metabolites in the blood and especially those concerning the concentration of glycogen in the liver indicate that during the development of resistance to trauma the experimental animal changes its internal environment. Trauma-resistant rats have a lower concentration of liver glycogen than control animals. In view of the fact that

resting values were determined on the 9th day after adaptation this decreased glycogen concentration cannot be due to the effect of the last traumatization during development of resistance. Similar data are given in the tables of Neufeld *et al.* (11) although these authors do not draw attention to those differences. The decreased level of liver glycogen in trauma-resistant rats is evidently not due to any inability of the liver to accumulate glycogen but is the result of a change in the regulation of glycogen formation. Soon after trauma (Figs. 2 and 3) glycogen levels in the liver of trauma-resistant rats rise above resting values. Results indicate that the liver of trauma-resistant animals reacts with a more rapid synthesis of glycogen as can be seen from the course of changes in liver glycogen contents after trauma.

The biphasic course of metabolic changes after trauma is evidently preserved in animals resistant to trauma. From the results of Neufeld *et al.* (11) and McShan *et al.* (9) it could be concluded that trauma-resistant animals differ from control animals only in the fact that their metabolic response is quantitatively less pronounced. These results, however, concerned changes only immediately after trauma (a maximum of 2 hours) and the results presented here are in agreement with their data. Thus during the first phase the metabolic response to trauma of trauma-resistant animals differs from that of control ones only quantitatively. During the second phase, however, lasting from 3 to 24 hours after injury, the metabolic response of trauma-resistant animals differs considerably from that of controls.

Evidently true metabolic adaptation after trauma is concerned with this second phase. Thus the level of pyruvic acid, which in control animals is again elevated during the 6th hour after trauma does not change in adapted rats. From this it may be concluded that during the second phase of metabolic changes after trauma, adaptation of the metabolism of pyruvic acid occurred. Manning and Hampton (8) pointed out that animals resistant to trauma show only a very slight decrease in oxygen consumption 4 hours after trauma, whereas oxygen consumption decreases considerably in control animals during the same period. In trauma-resistant animals urea also does not accumulate 3 hours after injury as it does in control animals. It is possible that the sudden rise in urea in control animals after injury is due to injury to kidneys. The metabolism of acetone bodies also differs in adapted animals. In trauma-resistant rats acetone bodies in the blood rise considerably, maximum values being observed 24 hours after injury. The question arises whether this rise in acetone bodies in the blood is due to increased fat mobilization developed during adaptation. This possibility is supported by the fact that in adapted animals nearly all subcutaneous fat disappears (unpublished observations).

Acknowledgment

The authors wish to thank Dr. O. Poupa for his interest and criticism. The valuable assistance of Mrs. M. Pochmanová, Miss D. Schmidová, and Mr. J. Veselý is gratefully acknowledged.

References

1. CHYTIL, F. J. physiol. Paris, **48**, 450 (1956).
2. CONWAY, E. J. and O'MALLOY, E. Biochem. J. **36**, 655 (1942).
3. FRAME, E. G., RUSSEL, J. A., and WILHELMI, A. E. J. Biol. Chem. **149**, 255 (1943).
4. GOOD, C. A., KRAMER, H., and SOMOGYI, M. J. Biol. Chem. **100**, 485 (1933).
5. HRUZA, Z. and CHYTIL, F. Physiol. Bohemosloven. In press.
6. HRUZA, Z. and POUPA, O. Physiol. Bohemosloven. **6**, 179 (1957).
7. LE PAGE, G. A. Am. J. Physiol. **146**, 267 (1946).
8. MANNING, J. W., JR. and HAMPTON, J. K., JR. Am. J. Physiol. **188**, 99 (1957).
9. MCSHAN, W. H., POTTER, V. R., GOLDMAN, A., SHIPLEY, E. G., and MEYER, R. K. Am. J. Physiol. **145**, 93 (1945–46).
10. NELSON, N. J. Biol. Chem. **153**, 375 (1944).
11. NEUFELD, A. H., TOBY, C. G., and NOBLE, R. L. Proc. Soc. Exptl. Biol. Med. **54**, 249 (1943).
12. NOBLE, R. L. Am. J. Physiol. **138**, 346 (1943).
13. NOBLE, R. L. and COLLIP, J. B. Quart. J. Exptl. Physiol. **31**, 187 (1942).
14. PLACER, Z. and PARIZEK, J. Čs. gastroenterologie a výživa, **10**, 200 (1956).
15. SLAVIK, K. and MICHALEC, C. Chem. listy, **43**, 102 (1949).
16. THIN, C. and ROBERTSON, A. Biochem. J. **5**, 218 (1952).

PHYSIOLOGY, BIOCHEMISTRY AND PHARMACOLOGY

A comprehensive monthly abstracting publication of Excerpta Medica covering the world's medical literature, fully classified under the following major headings: Biochemistry; Nutrition; Metabolism; Digestive System; Respiration; Circulation; Body Fluids; Excretion; Organs of Movement; Nervous System; Sense Organs; Reproduction; Aviation Physiology; Skin; General Physiology; Toxicology; Special and General Pharmacology.

1300 pages containing approximately 5700 abstracts a year

Price: $45.00 a year

EXCERPTA MEDICA FOUNDATION

N.Y. Academy of Medicine Building, 2 East 103rd St.,
New York 29, N.Y.

CANADIAN JOURNAL OF BIOCHEMISTRY AND PHYSIOLOGY

Notes to Contributors

Manuscripts

(i) ***General***

Manuscripts, in English or French, should be typewritten, double spaced, on paper 8½ × 11 in. **The original and one copy are to be submitted.** Tables and captions for the figures should be placed at the end of the manuscript. Every sheet of the manuscript should be numbered.

Style, arrangement, spelling, and abbreviations should conform to the usage of recent numbers of this journal. Names of all simple compounds, rather than their formulas, should be used in the text. Greek letters or unusual signs should be written plainly or explained by marginal notes. Superscripts and subscripts must be legible and carefully placed.

Manuscripts and illustrations should be carefully checked before they are submitted. Authors will be charged for unnecessary deviations from the usual format and for changes made in the proof that are considered excessive or unnecessary.

(ii) ***Abstract***

An abstract of not more than about 200 words, indicating the scope of the work and the principal findings, is required, except in Notes.

(iii) ***References***

These should be designated in the text by a key number and listed at the end of the paper, with the number, in the order in which they are cited. The form of the citations should be that used in this journal; in references to papers in periodicals, titles should not be given and initial page numbers only are required. The names of periodicals should be abbreviated in the form given in the most recent *List of Periodicals Abstracted by Chemical Abstracts*. All citations should be checked with the original articles.

(iv) ***Tables***

Tables should be numbered in roman numerals and each table referred to in the text. Titles should always be given but should be brief; column headings should be brief and descriptive matter in the tables confined to a minimum. Vertical rules should not be used. Numerous small tables should be avoided.

Illustrations

(i) ***General***

All figures (including each figure of the plates) should be numbered consecutively from 1 up, in arabic numerals, and each figure should be referred to in the text. The author's name, title of the paper, and figure number should be written in the lower left-hand corner of the sheets on which the illustrations appear. Captions should not be written on the illustrations (see Manuscripts (i)).

(ii) ***Line drawings***

Drawings should be carefully made with India ink on white drawing paper, blue tracing linen, or co-ordinate paper ruled in blue only; any co-ordinate lines that are to appear in the reproduction should be ruled in black ink. Paper ruled in green, yellow, or red should not be used. All lines should be of sufficient thickness to reproduce well. Decimal points, periods, and stippled dots should be solid black circles large enough to be reduced if necessary. Letters and numerals should be neatly made, preferably with a stencil (**do NOT use typewriting**), and be of such size that the smallest lettering will be not less than 1 mm. high when reproduced in a cut 3 in. wide.

Many drawings are made too large; originals should not be more than 2 or 3 times the size of the desired reproduction. Wherever possible two or more drawings should be grouped to reduce the number of cuts required. In such groups of drawings, or in large drawings, full use of the space available should be made; the ratio of height to width should conform to that of a journal page (4¾ × 7½ in.), but allowance must be made for the captions.

The original drawings and one set of clear copies (e.g. small photographs) are to be submitted.

(iii) ***Photographs***

Prints should be made on glossy paper, with strong contrasts. They should be trimmed so that essential features only are shown and mounted carefully, with rubber cement, on white cardboard, with no space between them. In mounting, full use of the space available should be made to reduce the number of cuts required (see Illustrations (ii)). Photographs or groups of photographs should not be more than 2 or 3 times the size of the desired reproduction.

Photographs are to be submitted in duplicate; if they are to be reproduced in groups one set should be mounted, the duplicate set unmounted.

Reprints

A total of 50 reprints of each paper, without covers, are supplied free. Additional reprints, with or without covers, may be purchased at the time of publication.

Charges for reprints are based on the number of printed pages, which may be calculated approximately by multiplying by 0.6 the number of manuscript pages (double-spaced typewritten sheets, 8½ × 11 in.) and including the space occupied by illustrations. Prices and instructions for ordering reprints are sent out with the galley proof.

Any reprints required in addition to those requested on the author's reprint requisition form must be ordered officially as soon as the paper has been accepted for publication.

Contents

Page

E. Bilinski and W. B. McConnell—Studies on wheat plants using C^{14} compounds. VII. Utilization of pyruvate-2-C^{14} 381

F. J. Joubert and W. H. Cook—Separation and characterization of lipovitellin from hen egg yolk 389

F. J. Joubert and W. H. Cook—Preparation and characterization of phosvitin from hen egg yolk 399

A. S. V. Burgen—The secretion of lithium in saliva 409

P. A. Anastassiadis and R. H. Common—Liberation of hexosamine, hexuronic acid, and hydroxyproline from tissues by resin hydrolysis 413

Sydney M. Friedman, William A. Webber, Harold F. Scherrer, and Constance L. Friedman—Changes in salt and water distribution, blood pressure, and adrenal activity following neurohypophyseal denervation in the rat . . . 425

J. D. Wood and B. B. Migicovsky—The effect of dietary oils and fatty acids on cholesterol metabolism in the rat 433

A. S. Holt—The phase test intermediate and the allomerization of chlorophyll *a* 439

F. Chytil and Z. Hruza—Metabolic changes in trauma-resistant rats following trauma 457